

Martin Levine

Topics in Dental Biochemistry



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In memory of my brother Ian J Levine BDS 1947–2009

Preface

Over the last 30 years, the development of molecular biology has revolutionized our understanding of the biochemistry underlying biology and medicine. As yet, there is no introductory text that relates this revolution to topics of major interest to dentistry. Because of increasing demands to make biochemistry useful by translating its findings into better treatments for problems in medicine, the dental field needs a similar textbook. The primary aim of this book is to integrate general biochemistry into topics that specifically pertain to dental health and disease. First and second year dental students have completed a general biochemistry course, but have, at best, a sketchy idea of how the material in that course relates to dentistry. In a traditional dental curriculum, the topics of this book are covered in physiology, nutrition, anatomy, histology, microbiology or immunology. This book was written to enable dental students to integrate their general biochemistry within these topics of dental interest. It was considered neither desirable nor practical to fill the text with references, except for the figures and tables.

The formal discipline of dentistry was initially developed in the late 19th century to treat dental caries, but it quickly spread to treat all diseases that affect the oral cavity. Dental treatments have progressed enormously over the last 40 years, as have treatments for many other diseases. The most powerful new dental treatments have come from water fluoridation, better oral hygiene measures, new mechanical or replacement materials, and the adoption of drugs developed for non-dental diseases. Nevertheless, these measures are not universally effective and improvements can be made in many areas.

The most widespread and commonly treated dental diseases, dental caries and periodontal disease, are chronic conditions caused by interactions between the host and oral bacteria that are still only partially understood in detail. A second aim of this book is therefore to point out the current knowledge for a future generation to build upon. While I hope the descriptions of dental caries and fluoride are pretty standard, describing a modern and coherent view of periodontal disease was a problem. This is a field with which I began my PhD and am still active. Unfortunately, almost every researcher in this field has their own view of how chronic periodontitis begins and some may choose to disagree strongly with parts of Chapters 13 and 14. In these chapters, I have attempted to describe a *coherent biochemical view* of the development and progression of the various chronic and aggressive periodontal diseases. A draft version of these chapters was reviewed by a colleague, Dr. Thomas Van Dyke, newly appointed Vice President of Clinical Research and Chair, Department of Periodontology, at The Forsyth Institute,

Boston. Tom gave me valuable insights on how to draft these chapters, but the end product is mine.

I am indebted to the Oklahoma College of Dentistry Faculty, Dean Steven Young and Dr. Kenneth Coy, for encouraging me to develop this book, which is based on my lectures to first-year dental students during their second semester. I very much thank Dr. Celeste Wirsig, Associate Professor, Dept of Cell Biology, University of Oklahoma Health Sciences Center (OUHSC), for reading and re-reading almost all of the many draft chapters, and for figures credited to her; Dr. Paul DeAngelis, professor and colleague in the Department of Biochemistry & Molecular Biology, University of Oklahoma Health Sciences Center, who contributed substantially to the chapter on blood clotting; Dr. Chadwick Cox who first sketched the figures that Dr. DeAngelis provided for this book; and Ms Lindsay Collins, my technical assistant, who tirelessly reformatted all the chapters and helped me negotiate copyright approval for figures and tables as necessary.

I would also like to thank following who reviewed proofs: Dr. Sharon M. Wahl, Chief, Oral Infection and Immunity Branch and some of her staff at NIDCR who provided me with helpful suggestions and comments on many of the chapters; OUHSC Graduate Students in Biochemistry and Dentistry, Mary Tappert (Chapters 1 and 2) and John R Lovell (Chapters 12 and 16); Dr. Zsolt Lohinai, a colleague at the Department of Conservative Dentistry, Institute of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary (Chapter 13); Dr. Augén Pioszak, an expert in calcium metabolism and a new colleague in the Department (Chapters 19 and 10), Dr. DeAngelis (Chapter 11) and Dr. Wirsig-Weichmann (Chapters 3 through 8).

I wish to thank the Springer Verlag Editorial Board for agreeing to publish this book, and their assistants who asked me every year when the book would be ready and who gave me innumerable deadlines that I could not keep. I hope very much that this book fulfils their expectations.

Finally, I dedicate this book to my wife, Laura, for her continuous support of my career. I began my career as a BDS degree and was working as a Dentist in the UK National Health Service alongside my father in Glasgow, Scotland at the age of 23. Laura encouraged me to follow my dreams and undertake a BSc honors degree in Biochemistry, followed by a PhD degree from the University of Glasgow. She and our two very young boys accompanied me for a year in the USA on a Sir Henry Wellcome Fellowship at the University of Washington, Seattle in 1973. The following year, I was invited to become a visiting assistant professor at SUNY Buffalo, where I started to teach the material in this book. In 1976, I came to the Dept of Biochemistry and Molecular Biology at the University of Oklahoma Health Sciences Center where I have spent the last 34 years.

Oklahoma, USA

Martin Levine

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Necessary Basics: Elements, Isotopes, Ions, Chemical Reactions, Energy Metabolism, and Bacterial Structures

1

This chapter describes how a traditional discussion of atomic structure and its relationship to organic chemistry is relevant to dentistry. Although the fundamental chemistry and biochemistry described here are common knowledge, the discussion centers on the associations with teeth and dental disease (Sects. 1 and 2). In addition, the difference between respiration and fermentation and the different types of fermentations involved in dental caries and periodontal disease are discussed (Sect. 3). The chapter concludes with a discussion of the difference between the outer cell surface of gram-positive and gram-negative bacteria that is important in causing these diseases (Sect. 4).

1.1.1.

Atomic Structure: Elements and Isotopes

Matter is made up of atoms, which are composed of protons, neutrons, and electrons. Protons are positively charged particles, electrons are negatively charged particles, and neutrons are uncharged particles. Neutrons glue the protons together and prevent the positively charged protons from repelling each other and destroying the nucleus. The positively charged atomic nucleus is electrically neutral because it is surrounded by a number of negatively charged electrons equal to the number of protons. Electrons are insignificant with respect to nuclear mass but provide chemical reactivity. The number of protons (atomic number) defines the elements of the chemical periodic table (Fig. 1.1) because it determines the number of electrons.

The nucleus of the first element, hydrogen, consists only of a proton. The second, helium, has two protons and two neutrons. The third, lithium, has three protons and three neutrons, and so on (Fig. 1.2). The atomic weight, measured as daltons (Da), is the sum of the number of protons and neutrons in an element's atomic nucleus. Hydrogen has a mass of 1 Da (the mass of a proton). Helium has two protons and two neutrons to hold the protons together. It therefore has a mass of 4 Da. Lithium has three protons and three neutrons and therefore a mass of 6 Da (Fig. 1.3). Oxygen has an atomic number of 8 and atomic mass of 16.

Isotopes are elements possessing different numbers of neutrons. Deuterium, a hydrogen atom containing a neutron in addition to a proton, has the same chemical properties as

Group #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Period																		
1	1 H																	2 He
2	3	4											5 B	6 C	7 N	8 O	9 F	10
3	11 Na	12 Mg											13 Al	14 Si	15 P	16 S	17 Cl	18
4	19 K	20 Ca			23 V	24 Cr	25 Mn	26 Fe	27 Co	28 Ni	29 Cu	30 Zn	31 Ga	32	33 As	34 Se	35 Br	36
5						42 Mo						48 Cd					53 I	54
6						74 W												

Fig. 1.1 Periodic table showing the elements in the major groups and transition metals. Only the elements associated with life are listed. The common elements are in *dark green*. *Light orange/yellow* or *pink/white* backgrounds indicate the metal and halide elements that occur as ions. Trace elements are indicated in *red*, *pink*, or *light green* (Modified from the full Periodic Table found in Wikipedia at http://en.wikipedia.org/wiki/Periodic_table)

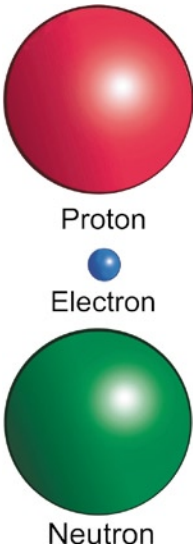


Fig. 1.2 Proton, neutron and electron

Fig. 1.3 Atomic composition of hydrogen, helium, and lithium, the first three elements of the periodic table. (Diagram prepared by Dr Wirsig-Weichmann)

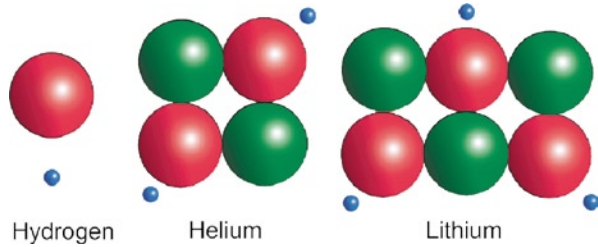
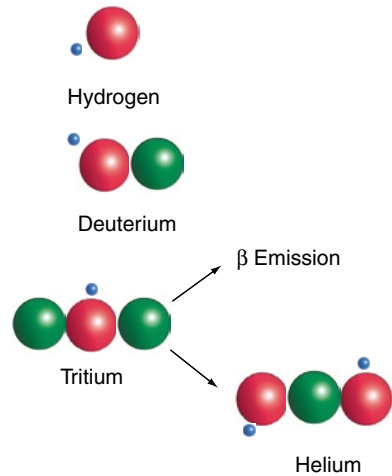


Fig. 1.4 Isotopes of hydrogen. Tritium is unstable. It changes to helium by a neutron emitting a β -particle and becoming a second proton. The β -particle is a radioactive emission. (Diagram prepared by Dr Wirsig-Weichmann)



hydrogen but twice the atomic mass (heavy hydrogen), 2 Da instead of 1 (Fig. 1.4). There is also a rarely occurring third isotope of hydrogen in nature, tritium. Tritium is important because it is radioactive; one of its neutrons is unstable and releases a beta (β) particle. Beta particles resemble fast-moving, free electrons that may be positively charged (positrons), or more commonly negatively charged (electrons). The release of a β -particle converts one of the two neutrons of tritium (Fig. 1.3) to a proton, making a stable isotope of helium (two protons and one neutron, Fig. 1.4). Table 1.1 lists the radioactive elements important in biology, the nature of the emitted radioactivity, what element they decay to, and their usual use in biology.

By the third decade of the twentieth century, the emitted radioactivity from artificially synthesized tritium (^3H) and carbon-14 (^{14}C) was used to follow biochemical reactions. Glucose, amino acids, and other chemical compounds were synthesized with ^3H or ^{14}C incorporated either randomly or at some known position in the molecule. The radioactive products derived from radiolabeled glucose or amino acids added to cells, tissue slices, or a whole organism identified the metabolic fate of these molecules under defined

Table 1.1 Radioactive isotopes important in biology

Element	Symbol	Isotope	Symbol	Decays to	Symbol	Primary Use
Hydrogen	¹ H	^a Tritium	³ H	Helium ^b	³ He	Metabolic pathways
Carbon	¹² C	^a Carbon-14	¹⁴ C	Nitrogen	¹⁴ N	Metabolic pathways
Phosphorus	³¹ P	^c Phosphorus-32	³² P	Sulfur	³² S	Phosphorylations
Calcium	⁴⁰ Ca	^a Calcium-41	⁴¹ C	Potassium	⁴¹ K	Bone metabolism
Iodine	¹²⁷ I	^a Iodine-131	¹³¹ I	Xenon	¹³¹ Xe	Thyroid cancer therapy
Iodine	¹²⁷ I	^d Iodine-125	¹²⁵ I	Telurium-125 ^d	¹²⁵ Te	Protein labeling

Notes:

^aWeak β-emission

^bRare isotope

^cStrong β-emission

^dEmits weak gamma rays giving an unstable isotope of ¹²⁵Te that stabilizes by capturing a photon and emitting an Xray

conditions. These studies led to the establishment of the metabolic pathways that we now take for granted such as protein, fatty acid, amino acid, and nucleic acid metabolism.

1.1.2.
Isotopes Date Paleontology Samples Such as Teeth

Another use of radioactive carbon-14 is to date prehistoric bones and teeth, the longest-surviving parts of the body. Carbon-14 (¹⁴C; six protons and eight neutrons) is continually formed in the upper atmosphere by cosmic rays acting on nitrogen (¹⁴N; seven protons and seven neutrons). The energy of the cosmic rays causes a proton and electron in atoms of ¹⁴N to fuse into a neutron. The newly formed ¹⁴C is rapidly oxidized to ¹⁴CO₂, which enters the earth’s plant and animal life through photosynthesis and the food chain. The ratio of ¹⁴C to nonradioactive carbon is approximately constant over time. When plants or animals die, carbon uptake ceases, ¹⁴C is not replenished, and it slowly decays with a half-life of close to 5,700 years. The radioactivity of a sample whose age is not known can be used to indicate the amount of ¹⁴C remaining, provided it is not more than 40,000 years of age. After that time, so much ¹⁴C has decayed that what is left is not measurable. From the ratio of radioactive carbon to total carbon content, it is possible to determine how much was lost relative to the current ratio (unchanged over billions of years) and therefore how long ago the bone or tooth was part of a living organism.

1.1.3.

Isotopes Indicate Ancient Life Forms and Climate Changes

Not all isotopes are radioactive, and even some stable isotopes can be useful. A stable isotope of carbon, carbon-13 (^{13}C ; 6 protons and 7 neutrons) has accounts for about 1% of carbon atoms on earth. Because it is chemically more stable than ^{12}C , living organisms preferentially utilize ^{12}C for chemical reactions (metabolism). Therefore, rocks containing a greater than usual $^{12}\text{C}/^{13}\text{C}$ ratio are potentially ‘chemical fingerprints’ of life. Minute residues in some of the oldest rocks on Earth, from Akilia Island near Greenland, have a chemical fingerprint that may have come from living organisms. Analysis of selected tiny samples using an ion microprobe revealed ratios of carbon-13 to carbon-12 that were 2–5% less than expected. Some prebiotic process may have enriched the rock with carbon-12 atoms, and it lay apparently undisturbed (based on the surrounding crystal structure) for over 3,700 million years (3.7 Giga-years, Gyr). The age of ancient rock samples is independently determined from the rate of decay of another isotope, ^{87}Rb (Rubidium) to ^{87}Sr (Strontium). The half-life of this decay is about 1.0 Gyr. Amounts of ^{87}Sr and ^{87}Rb are measured, and a ratio is derived and compared with the ratio of ^{87}Sr to stable strontium (^{86}Sr) in the rock sample. The age of the rock sample is then derived mathematically from these ratios, given the half-life of ^{87}Rb .

The common element of oxygen has eight protons and eight neutrons. A stable isotope with two additional neutrons is also relatively common. Because water-containing ^{18}O evaporates more slowly and condenses more rapidly, the ratio of $^{18}\text{O}/^{16}\text{O}$ in Antarctic ice cores indicates major climate shifts since early in geologic time. Increased ^{18}O indicates a sudden warming, and increased ^{16}O indicates a sudden cooling.

1.1.4.

The Elements in Biology

The electrons of the elements are arranged in shells surrounding the nucleus. The first shell consists of two electrons, the second and third each of eight electrons, and the fourth and fifth each of 18 electrons. Elements take part in a chemical reaction by gaining or sharing electrons to complete their outer shell except for the noble gases (helium, neon, argon, krypton and xenon, far right-hand column of the periodic table). These elements already have a complete outermost electron shell. They therefore have no chemical reactivity and are incompatible with life.

Figure 1.1 shows the elements important for all of life. The common elements are depicted in dark green boxes: carbon, hydrogen, nitrogen, oxygen, phosphorous, and sulfur. Elements depicted in light orange/yellow or pink/white boxes make up the major cations and anions of living organisms: sodium, potassium, magnesium, calcium, manganese, iron, cobalt, nickel, copper, zinc, chlorine, and iodine. Trace elements (light green, pink, or red boxes) occur as occasional enzyme cofactors: boron, fluorine, silicon, arsenic, selenium and bromine, aluminum, gallium, chromium, vanadium, molybdenum, tungsten, and cadmium.

1.1.5.

Fluorides

Fluorine is present on earth only as *fluoride*, a negatively charged anion that is especially important in dentistry because of its ability to mediate protection from dental caries (Chapter 16, section 16.2.1.). Although metabolites of chlorine and iodine are ubiquitous, few biological products contain fluoride because of its tight hydration shell. The few enzymes that utilize fluoride as a cofactor must overcome an exceptionally high desolvation energy barrier.

The fluoride content of a solution is measured with an electrode made from a mixture of lanthanum and europium fluorides. Lanthanum follows barium in the periodic table and has an atomic number of 57. Lanthanum is also the first of a series of 15 heavy metals before hafnium called lanthanides. Europium is a lanthanide and its atomic number is 63. The mode of action of the fluoride electrode is described in Chapter 16, sect. 16.1.1.

Matter consists of atoms that are made up of protons (electropositive), electrons (electronegative), and neutrons (electrically neutral). Because the number of electrons and protons is equal, elements, atoms with different numbers of protons, have different numbers of electrons. The chemical properties of an element depend on the number of electrons, but because the electrons have almost no mass, the atomic weight of an element is its number of protons and neutrons. Neutrons are needed to hold the protons together in the nucleus. Isotopes are elements with different numbers of neutrons. Isotopes with too many neutrons are unstable and emit radioactivity. Radioactive and nonradioactive isotopes are used to follow biochemical reactions in health and disease, to date paleontology specimens, usually bones and teeth, and detect traces of life in ancient rocks.

1.2.1.

Chemical Bonds

Four chemical bonds are important in living organisms: electrostatic bonds, covalent bonds, polarized covalent bonds, and hydrophobic bonds.

1.2.2.

Electrostatic Bonds

Electrostatic bonds usually form ions; sodium chloride and potassium chloride are examples of ionic compounds. Sodium (or potassium) has a single electron in its outer

shell, whereas chlorine is missing an electron in its outer shell. The salt, sodium chloride, is an electrostatic compound consisting of sodium ions, sodium atoms that have donated an electron and become positively charged (cations), and chloridions, chlorine atoms that have received an electron and become negatively charged (anions).

Most electrostatically bonded solids form crystals. In sodium chloride, the sodium cations and chloride anions form an electrically neutral square – a crystal cell. This simple shape causes sodium chloride crystals to be granular. By contrast, calcium phosphate is a more complex ion pair, and it has a correspondingly more complex crystal structure (apatite). Apatite forms the mineralized structures of bones and teeth – a hard, smooth agranular surface. Calcium forms cations by losing two electrons. Phosphate is not an elemental anion; it contains four oxygen atoms that are covalently bound to a central phosphorus atom. Phosphates have one, two, or three negative charges (monovalent, divalent, or trivalent) depending on the pH of the surrounding solution. As the pH of the solution increases, so also does the net charge of the phosphate ion, causing a calcium phosphate precipitate to undergo intramolecular rearrangements that produce apatite and decrease solubility (see Chapter 9: [Sect. 9.1.2](#)).

Amorphous solids including proteins have no crystal cell, but may crystallize and precipitate under appropriate conditions. The repeating crystal cell provides an x-ray diffraction pattern that can provide a detailed 3-dimensional protein structure. To be crystallized, a protein must be pure and in solution. It is then tested for crystal formation by very slow evaporation under a variety of pH and ionic strength conditions.

1.2.3. Covalent Bonds

Carbon has 4 electrons in its outermost shell. Removing all 4 would make the atom too positively charged to be stable. Conversely, adding 4 electrons would make the atom too negatively charged to be stable. Because the formation of carbon ions is not energetically feasible, carbon shares its electrons by forming covalently bonded molecules. Each electron points in a different geometrical direction so that each bond of a carbon atom points in a different direction. Although many different elements can share electrons with carbon, the ones depicted in green in [Fig. 1.1](#) are preferred by biological systems.

1.2.4. Polarized Covalent Bonds

Polarized covalent bonding, is evident in water. Because oxygen has six electrons in its outer shell, the electron donated by each hydrogen atom is pulled towards the oxygen atom to try and complete the latter's outer shell of eight electrons. The electrons are therefore unevenly

distributed around each water molecule. They are polarized to the oxygen atom, giving it a slight negative charge, and the hydrogen atoms a slight positive charge. Water is a fluid because the polarized molecules attract each other without forming a solid at atmospheric temperatures above 0°C and condense back from a gas to the liquid form at temperatures below 100°C. The polarization also attracts the ions in solids. The anions and cations to dissociate in the liquid and the solid dissolves forming a solution.

Carbon/oxygen bonds resemble hydrogen/oxygen bonds. In carbon dioxide, the two oxygen atoms are negatively charged and the carbon atom is positively charged. The arrangement resembles water, except that the positive charge is on the central carbon atom and the negative charge on the two oxygen atoms is on the outside. Carbon dioxide is less polarized than water and is therefore a gas, although it liquefies easily under pressure. The bonding of carbon to a single oxygen atom, as in an alcohol, ketone, aldehyde, or carboxylic acid, is more electrically withdrawing (polarizing), which provides greater water solubility and a point of attack for metabolic reactions.

Nitrogen and sulfur bonding also withdraw electrons from a carbon atom, causing it to become polarized like oxygen/carbon bonds and provide additional sites for metabolic reactions. Nitrogen withdraws electrons less strongly than oxygen, whereas carbon-halide bonds are more electron withdrawing, but do not form biologically. As elements move from the 5th to the 6th and 7th column of the periodic table (Fig. 1.1), their electron-withdrawing power increases when bonded to carbon.

1.2.5. Hydrophobic Bonds

The electrons in hydrogen – carbon bonds are evenly shared, leaving a molecule completely uncharged (apolar) and insoluble in water. In large molecules composed mostly of carbon-hydrogen bonds but with a small polarized region such as a carboxylic acid at one end as in a fatty acid, the hydrocarbon regions clump together. If the fatty acids are part of a phospholipid, the hydrocarbon regions form the interior of membranes that delineate cells from their environment. In proteins, regions of hydrocarbon promote hydrophobic regions that attract each other. Together with charged and polarized regions caused by carbon bound to oxygen, nitrogen and sulfur atoms, the hydrophobic attraction promotes folding and protein-protein and/or enzyme-substrate interactions.

Molecules are composed of atoms that are attached to each other by sharing electrons to complete their respective electron shells. Electrostatic bonds arise when electrons are added or subtracted from elements, giving rise to positive and negatively charged particles called ions. Covalent bonds do not give rise to ions but may be polarized as a consequence of the electron-withdrawing properties of adjacent atoms in the molecule, permitting interactions with water, the solvent for biochemical reactions. Polar bonds are water soluble, whereas apolar bonds (mostly carbon–hydrogen) are water insoluble (hydrophobic).

1.3.1.

Mechanisms of Energy Production: Respiration and Fermentation

All living organisms get their chemical energy from ATP and a hydride: a reduced form of nicotinamide adenosine nucleotide diphosphate, $\text{NADH} + \text{H}^+$, or its phosphorylated analog, $\text{NADPH} + \text{H}^+$ (Fig. 1.5).

Organisms obtain ATP and NADH in one of three ways:

1. Respiration (conversion of ingested foods to CO_2 and H_2O)
2. Fermentation (partial oxidation of carbon compounds)
3. Photosynthesis (action of sunlight on water and CO_2 in chloroplasts, Chap. 2)

In respiration, substrate organic molecules containing carbon–hydrogen bonds (food) and oxygen are absorbed by prokaryotic cells or by the mitochondria of eukaryotic cells. The oxygen reacts with electrons that are derived from metabolic changes to the carbon–hydrogen bonds of the substrates. The final steps of substrate metabolism, the Krebs cycle,

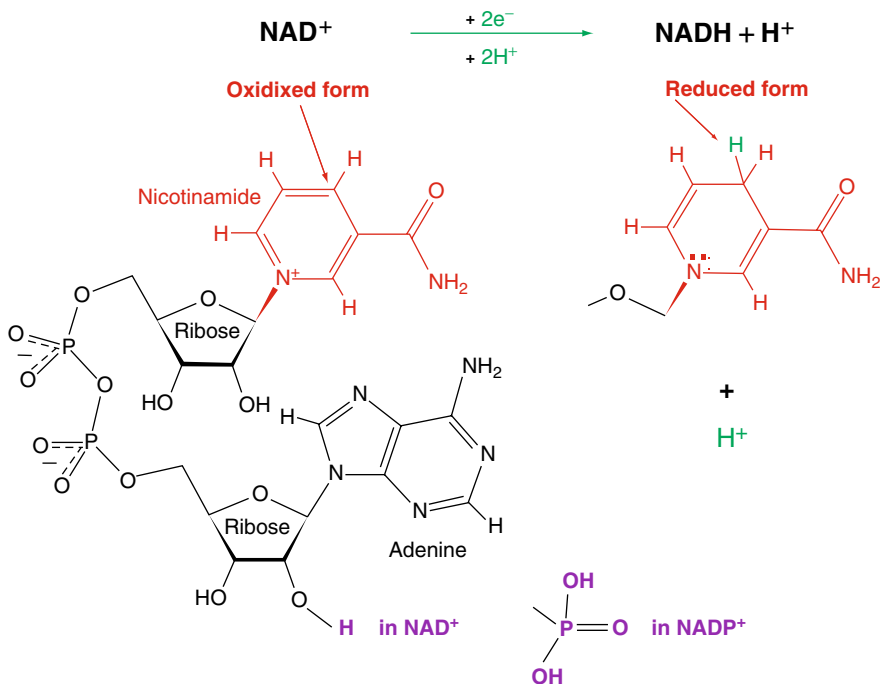
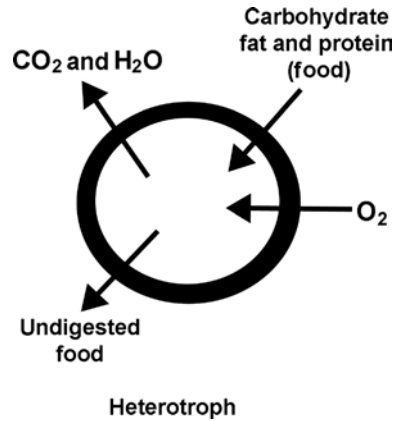


Fig. 1.5 Nicotinamide adenine dinucleotide (NAD^+) and its phosphorylated analog (NADP^+). The difference is indicated in purple. NAD^+ and NADP^+ undergo reduction to NADH and NADPH by accepting a hydride ion and two electrons and releasing a proton from an oxidized substrate. (Adapted from Fig. 14-13 in Berg JM, Tymoczko JL and Stryer L. Biochemistry, 5th Ed. 2002. W.H. Freeman & Co., New York)

Fig. 1.6 Respiration. Energy is obtained by converting foodstuffs (organic material composed mainly of carbohydrate, fat, and protein) to oxygen and water. These organisms obtain the food from many sources and are called heterotrophs



produce carbon dioxide, NADH, and protons. The electrons are transferred from NADH to oxygen along with protons, forming water and ATP. The water and carbon dioxide are excreted, and the ATP is used intracellularly with some of the NADH for cell maintenance and growth. In vertebrates, the oxygen and carbon dioxide are respectively absorbed from and excreted to the environment through the circulation of hemoglobin between the lungs and tissues (Fig. 1.6).

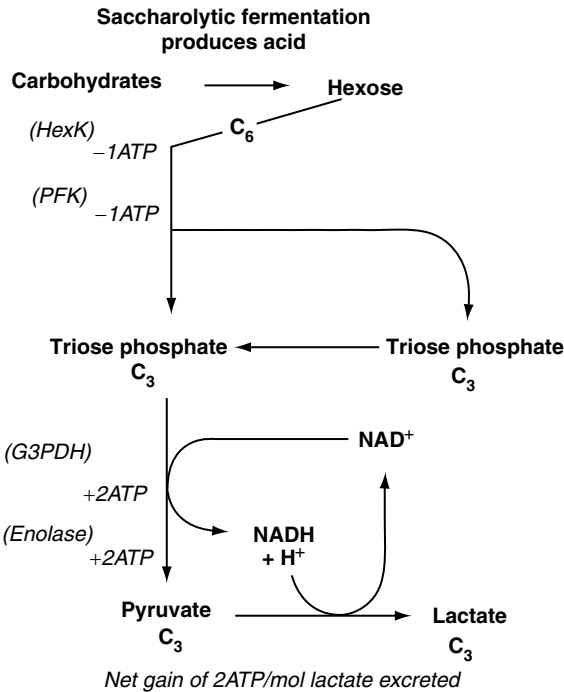
In fermentation, energy is obtained by shuffling organic molecules so that NADH produced in one step is oxidized by a subsequent product or products, which are reduced and excreted. Due to the small amount of energy obtained, only microbes can rely on fermentation as their sole energy source. More complex organisms utilize respiration alone or in combination with sunlight.

Dental caries results from microbial fermentations that produce lactate by glycolysis from monosaccharides following the hydrolysis of disaccharide and polysaccharides (Sect. 15.2.2). These bacteria are commonly referred to as *saccharolytic* (sugar metabolizing) to distinguish them from *asaccharolytic* (nonsugar metabolizing) bacteria, most of which hydrolyze proteins and utilize the amino acids for energy. A third type of fermentation is the use of an inorganic molecule such as nitrate as electron acceptor. The nitrate is reduced to nitrite by electron transport in a manner similar to the reduction of oxygen in respiration, but only 1 mol of ATP is produced per mol of nitrate. Reducing oxygen produces 2–3 mol of ATP per mol of water (Fig. 1.7).

1.3.2.

The Oral Microbiota, Dental Caries, and Periodontal Disease

Teeth adherent *bacterial biofilms*, commonly called *plaque* or *plaques*, are responsible for the common forms of periodontal disease (Chap. 13) and dental caries (Chap. 15). In children or adults who keep their teeth clean and have no periodontal disease, the bacteria in a biofilm (the *microbiota*) is mostly gram positive and resembles that in saliva or adhering to the oral mucosa. The microbiota is predominantly *saccharolytic* and the major fermentation



NOTE: The mass of a molecule is identified by the sum of its atomic weights, the molecular weight equivalent (mole). The standard symbol for mole is 'mol'

Fig. 1.7 Saccharolytic fermentation produces acid. The conversion of glucose to lactic acid (glycolysis) is an example of fermentation. Adenosine nucleoside triphosphate (ATP) is synthesized and NADH is oxidized from carbon substrates without any need for oxygen. For example, bacteria adherent to teeth in the oral cavity may obtain energy from ingested carbohydrates by glycolysis (Chapter 15). Two ATP are used up to convert monosaccharide hexose to 2 molecules of 3-phosphoglycerate. One ATP is used by hexokinase (HexK) to make the hexose 6-phosphate and one by phosphofructokinase (PFK) to make fructose biphosphate. Two triose phosphate molecules are made from fructose biphosphate and both are converted to glyceraldehyde 3-phosphate (G3P). The G3P is acted on by its dehydrogenase (G3PDH) to produce 2 molecules of 3-phosphoglycerate, 2 ATP and 2 NADH. The 3-phosphoglycerate molecules are both converted to phosphoenolpyruvate (PEP), which provides 2 ATP when the two PEP molecules are converted to pyruvate by enolase. There are therefore a total of 4 ATP produced of which 2 are used up in converting hexose to triose, leaving a net gain of 2 ATP per hexose molecule for the organism. The 2 molecules of NADH are reoxidized to NAD^+ when the 2 molecules of pyruvate are converted to lactate. The lactate is extruded from the cell as lactic acid which decreases the pH and causes dental caries at the tooth surface (Chapter 15). (Figure is adapted from Fig. 1, Chapter 15, in Harper's Review of Biochemistry. D. W. Martin et al., 20th edition, 1985, Lange Medical Publications, Los Altos, CA)

end product is lactic acid. Whole saliva contains mucins, proteins covered with numerous saccharide (glycan) residues that are more accessible to enzymes than the polypeptide. In addition, the repeated intake of dietary carbohydrate predisposes to a *saccharolytic microbiota* (Sect. 15.1.4). These bacteria are predominantly gram positive and possess a thick

outer wall (Sect. 1.4.1). The thick cell walls enable some of these bacteria to tolerate the low pH caused by their production of large amounts of lactate, which causes caries by dissolving tooth enamel and dentin (Sects. 15.1.4 and 15.2.3).

By contrast, beneath a healthy gingival sulcus, there is an intermittent flow of proteins from serum, blood plasma proteins in which clotting has been inactivated (Sect. 11.4.1). This exudate of serum proteins, the gingival crevicular fluid (GCF), provides a sulcus that is richer in proteins than saliva and an environment that is more suited for an *asaccharolytic microbiota* (Sect. 13.1.2). Asaccharolytic bacteria secrete proteases that digest proteins to small peptides, which they digest (ferment) in the cytosol.

In asaccharolytic fermentations, amino acids are deaminated to ammonia in a reaction that converts NAD^+ to NADH: for example, the deamidation of glutamate to α -ketoglutarate and ammonia (Fig. 1.8). α -Ketoglutarate may then be decarboxylated and reduced to butyrate. Other amino acids are manipulated to produce short chain fatty acids such as formate, acetate, and propionate, which are excreted with a net gain of ATP to the organism. These reactions are bacteria specific and therefore extremely varied, but the common factor is ammonia production and short chain fatty acids whose products are toxic to mammalian cells (Sect. 13.4.1). Figure 1.8 illustrates relationships of glycine to acetate; of cysteine, alanine and aspartate to propionate; and of threonine and glutamate to butyrate. Despite the short chain fatty acids, ammonia accumulates enough to make the gingival sulcus alkaline. The high pH of the sulcus prevents caries from developing beneath the gingival sulcus (Sect. 15.3.2).

The alkaline environment also precipitates calcium and phosphate ions from the GCF, causing *dental calculus*. Dental calculus interferes with self-administered oral hygiene (Sect. 13.1.2) and asaccharolytic metabolism intensifies. When sulfur-containing amino acids (cysteine and methionine) are metabolized, they release hydrogen sulfide along with the ammonia and short chain fatty acids. Hydrogen sulfide is a major contributor of oral malodor that often accompanies moderate to severe periodontal disease.

Within the biofilms or plaques, different bacteria utilize each other's products in order to grow more efficiently (a mechanism called *symbiosis*). For example, a bacterium that metabolizes glucose by reducing it to lactate may enable another bacterium to grow by reducing the lactate to propionate. The formation of biofilms is driven by three factors: (1) the presence of substrate in saliva or gingival serum exudate for one or more of the bacteria in a symbiotic group; (2) the production of a metabolite such as lactate that can be utilized by other bacteria in the group, and (3) proteins and other components that permit the bacteria in the group to attach to each another.

Bacteria obtain energy by fermentation, in which shuffling carbon compounds produces ATP without a need for oxygen. NADH is produced, but reoxidized by the product of the shuffling, which is excreted. Bacteria ferment sugars and excrete lactate (saccharolytic), or ferment amino acids and excrete ammonia, sulfides, and short chain fatty acids (asaccharolytic). Fermentations may alternatively reduce an inorganic molecule such as nitrate to reoxidize their NADH. Nitrate is secreted by the salivary glands and some of the bacteria that normally infect saliva reduce the nitrate to nitrite by electron transport similar to the reduction of oxygen to water in respiration. This reduction provides only enough energy to produce 1 mol of ATP/mol nitrate, whereas the reduction of oxygen to water produces 3 moles. Saccharolytic

bacteria are associated with lactic acid production and dental caries, whereas asaccharolytic bacteria are associated with periodontal disease. The latter fermentations result in oral odor from sulfides and an alkaline environment from ammonia that is especially important in causing calculus to precipitate around the teeth as periodontal disease develops. The bacteria adhere to teeth as biofilms or plaques in which different bacteria utilize each other's products and grow better (symbiosis).

Asaccharolytic fermentation produces ammonia and short chain fatty acids	
$\text{CH}_3 - \text{COO}^-$	Acetate
$\text{NH}_2 - \text{CH}_2 - \text{COO}^-$	Glycine
$\text{CH}_3 - \text{CH}_2 - \text{COO}^-$	Propionate
$\begin{array}{c} \text{CH}_2 - \text{CH} - \text{COO}^- \\ \quad \\ \text{SH} \quad \text{NH}_2 \end{array}$	Cysteine
$\begin{array}{c} \text{CH}_3 - \text{CH} - \text{COO}^- \\ \\ \text{NH}_2 \end{array}$	Alanine
$\begin{array}{c} ^-\text{OOC} - \text{CH}_2 - \text{CH} - \text{COO}^- \\ \\ \text{NH}_2 \end{array}$	Aspartate
$\text{CH}_3 - \text{CH}_2 - \text{CH}_2 - \text{COO}^-$	Butyrate
$\begin{array}{c} \text{CH}_3 - \text{CH}_2 - \text{CH} - \text{COO}^- \\ \quad \\ \text{OH} \quad \text{NH}_2 \end{array}$	Threonine
$\begin{array}{c} ^-\text{OOC} - \text{CH}_2 - \text{CH}_2 - \text{CH} - \text{COO}^- \\ \\ \text{NH}_2 \end{array}$	Glutamate

Fig. 1.8 Asaccharolytic fermentation produces ammonia and short-chain fatty acids. This group of fermentations by oral bacteria utilizes proteins, which are converted to peptides and amino acids. The free amino acids are then deaminated to ammonia in a reaction that converts nicotinamide adenine dinucleotide (NAD) to NADH. For example, alanine is converted to pyruvate and ammonia. The pyruvate is reduced to lactate, and ammonium lactate is excreted into the environment. Unlike lactate from glucose, ammonium lactate is a neutral salt. The common end products in from plaque are ammonium acetate, ammonium propionate, and ammonium butyrate, ammonium salts of short chain fatty acids. For example, glycine is reduced to acetate and ammonia. Cysteine is reduced to propionate, hydrogen sulfide, and ammonia; alanine to propionate, water, and ammonia; and aspartate to propionate, carbon dioxide, and ammonia. Threonine is reduced to butyrate, water, and ammonia and glutamate is reduced to butyrate, carbon dioxide, and ammonia. Other amino acids are involved in more complicated metabolic reactions that give rise to these short-chain amino acids, sometimes with succinate, another common end product in plaque.

1.4.1.

Bacterial Cell Structures

The cell wall protects bacterial cells from the environment, just as skin or fur protects mammals. Its thickness distinguishes two major classes of bacteria: gram-positive staining (thick walled) and gram-negative staining (thin walled). The gram negative cell wall (blue in Fig. 1.9a but red when gram-stained) is composed of short peptides of D- and L-amino acids that are cross-linked by short glycan chains to form a *peptidoglycan* network containing other substances. The cell wall covers the outer surface of the plasma membrane from which fimbriae (singular: fimbria) and flagella (singular: flagellum) extrude (Fig. 1.9a, upper left side). A bacterium has many fimbriae, but only one or two flagella. *Penicillin* and related antibiotics inhibit an enzyme involved in synthesizing the cell wall peptidoglycan network. In addition, *lysozyme*, an enzyme in the acid-activated *lysosomal vesicles* of most mammalian cells (Sect. 10.1.1), hydrolyzes a repeating bond in the peptidoglycan polymer, breaking it up into small fragments. Both penicillin and lysozyme lyse a sensitive bacterial cell.

In periodontitis, bacterial cells that no longer survive in the gingival sulcus leave *peptidoglycan fragments* that may be absorbed into the cytosol of surrounding host cells. Within host cells, peptidoglycan fragments may either be toxic or cause a release of inflammatory mediators (Sect. 13.4.1).

1.4.2.

The Bacterial Outer Surface

Fimbriae, known also as pili (singular: pilus), are outer surface protein appendages that mediate adhesion, whereas *flagella* are a separate group of proteins responsible for motility. Fimbriae are very common. They are synthesized in the cytosol and assemble attached to the cell inner membrane. Many fimbrial proteins require secretion through the cell membrane, and they do so with the aid of an N-terminal peptide signal sequence and signal recognition particles containing small RNA resembling the small cytosolic RNA of eukaryotes (Type II or Type IV secretion). By contrast, flagellar proteins are synthesized in the cytosol and then interact with other proteins to translocate their extracellular components through the membrane. Flagella are assembled intra- and extracellularly around the cell membrane (Type III secretion). *Bacterial toxins* use these, or a fourth mechanism (Type I secretion described in Sect. 14.2.2), in order to reach the extracellular fluid and attack a target cell.

In many bacteria, the cell wall is surrounded by a *polysaccharide (glycan) capsule* through which the fimbriae and flagellum protrude. One end of the glycan may be covalently attached to fatty acids in the plasma membrane, or they may adhere by non-covalent bonds to the glycan-synthesizing enzyme within the plasma membrane. Capsules vary in content, composition, size and thickness, especially among gram-positive bacteria in which a capsule may be absent. A *capsule* made from *dietary sucrose* by the gram-positive bacterium, *Streptococcus mutans*, likely mediates *dental caries*.

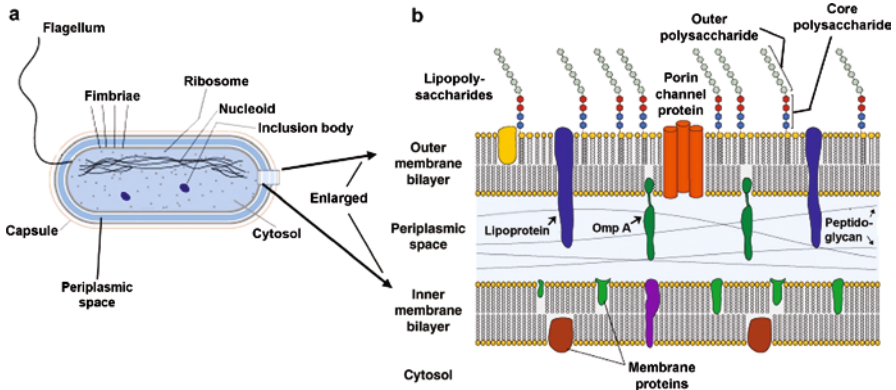


Fig. 1.9 Bacterial structure. **(a)** *Bacterial cell*. Bacteria have a simple internal structure with few organelles. They have no nucleus internally, but a nucleoid region where DNA is found. Plasmids are independent pieces of DNA that can be exchanged between cells and often carry genes that promote survival, for example genes encoding enzymes that remove antibiotics. The interior of the bacterial cell is full of ribosomes because the half-life of bacterial proteins is so short that they are constantly resynthesized. Bacterial proteins turn over constantly whereas many mammalian proteins turn over slowly. The exterior of the cell lies outside the plasma membrane. It consists of a cell wall and capsule. Fimbria and flagella extend from the plasma membrane and pass through the cell wall and capsule. In gram-negative bacteria, much of the cell wall is thinner than in gram-positive bacteria. Instead there is a second, outer membrane with an attached polysaccharide capsule. The space between the inner (plasma) and outer membranes is called the periplasmic space. In gram-positive cells, the periplasmic space is small and lies between the thick outer cell wall and the plasma membrane. Inclusion bodies are aggregates of viral proteins that may represent sites of multiplication or attempted multiplication of a bacteriophage (virus that infects bacteria). Inclusion bodies may occur in any bacterium, gram positive as well as gram negative. **(b)** *Structures of the double membrane, cell wall, and capsule of gram-negative bacteria*. Each membrane is a phospholipid bilayer. The inner membrane is the plasma membrane and separates the cytosol from the periplasmic space between the two bilayers. The major components of the periplasmic space are cell wall peptidoglycan, peptidoglycan-associated lipoprotein (lipoprotein), and outer membrane protein A (*omp A*). A similar double membrane structure is present in mitochondria but the protein composition of the intermembrane space is very different. Lipopolysaccharide (LPS) consists of an outer polysaccharide chain attached by a core polysaccharide to a lipid, which is part of the outer membrane (lipid A), shown as *darker regions* of the outer membrane. The precise composition of the outer and core polysaccharides and lipid A varies, with species and strain of the bacterium (Figure is modified from Wikipedia public domain: http://en.wikipedia.org/wiki/Bacterial_outer_membrane)

To make up for their thin peptidoglycan, gram-negative bacteria possess a second (outer) membrane to which is attached a glycan capsule called lipopolysaccharide (LPS). Figure 1.9a illustrates the inner and outer membranes of these bacteria. Figure 1.9b illustrates the detailed structure of the double membrane with the attached LPS. As its name suggests, LPS is composed of a polysaccharide that is covalently attached to a large complex lipid (lipid A) in the outer membrane, unlike the attachment of fimbriae or flagella, which is to the inner (plasma) membrane as in gram-positive bacteria. The polysaccharide portion of LPS is

composed of a core with side chains containing a variety of monosaccharides. The lipid A moiety usually contains unusual fatty acids.

As gram negative bacteria invade and grow within the dental biofilm, they shed LPS into the environment. The LPS penetrates the surrounding tissues and is recognized as foreign by mammalian cell surface receptors. This recognition may be important for activating gingival inflammation (gingivitis Sect. 13.2.1). LPS receptors are promiscuous in that they recognize almost all the various saccharides and lipids in LPS from different gram negative bacteria. Inhibiting gingival inflammation by inhibiting LPS receptor activation therefore seems impracticable. Although mechanically removing the dental biofilm by oral hygiene is the established method of controlling periodontal disease, it only works well in about 80% of patients. Chemical methods of inhibiting the colonization of dental biofilm by gram negative bacteria are discussed in Sect. 13.1.3.

Bacteria are simple unicellular organisms that constantly grow. They have a membrane and cell wall. Fimbriae are especially important for bacterial adhesion, a critical factor in dental disease development. Lipopolysaccharide is a covalent lipid and polysaccharide structure that contains unusual saccharides and fatty acids. The lipid is at one end and inserts it into the plasma membrane. LPS is invariably recognized as foreign by receptors on mammalian cells that recognize the unique structure and activate inflammation such as gingivitis.

Starch and sucrose, key substrates for the development of dental caries, are exclusively synthesized by plants. They are made in plant leaves by a process called photosynthesis, which utilizes sunlight as the energy source. This chapter outlines the light and dark reactions of photosynthesis and compares the light reaction with mitochondrial electron transport (Sect. 1). The key dark reaction, the production of phosphoglycerate by the enzyme ribulose biphosphate carboxylase (rubisco), is described along with the production of fructose, sucrose, and starch (Sect. 2). The chapter concludes with a detailed discussion of the roles of starch and sucrose in plant metabolism (Sect. 3).

2.1.1.

Role of Photosynthesis in Living Organisms

As discussed in Sect. 1.3.1, insects, animals, and bacteria respire by consuming oxygen gas and a complex mixture of organic material containing carbon–hydrogen bonds (proteins, fats, and carbohydrates) for energy, growth, and maintenance. By contrast, the photosynthetic organisms, plants and algae, consume carbon dioxide gas from which they make all their molecular carbon. Nitrogen-containing molecules (amino acids, nucleic acid bases, and various other compounds) require ammonia, which comes from decaying organisms in the soil, or from root-associated bacteria that produce it from nitrogen gas.

Photosynthesis splits water into hydrogen and oxygen atoms in a reaction that requires sunlight (light reaction). Oxygen is passed into the atmosphere and the hydrogen is used to assimilate carbon dioxide in a dark (non-photosynthetic) reaction that forms starch, sucrose, and another disaccharide called maltose. Metabolites such as α -ketoglutarate are derived from starch and sucrose and incorporate (fix) the ammonia absorbed from the soil into the nitrogen-containing compounds. The cycle is summarized in Fig. 2.1.

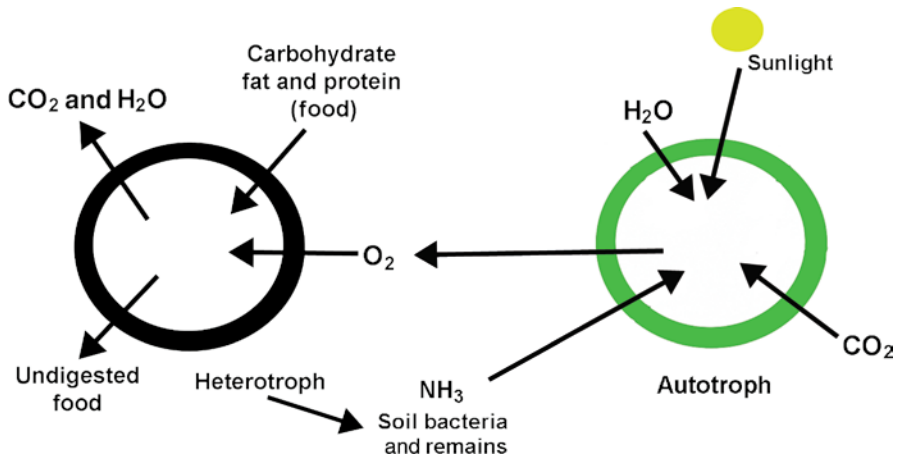


Fig. 2.1 Photosynthesis and respiration. *Left side* is Fig. 1.6. *Right side* shows photosynthesis in which sunlight and water in the atmosphere are absorbed by plants and algae to generate ATP and NADPH, which make carbohydrates and other organic carbon products from carbon dioxide, which is absorbed from the atmosphere separately. All of the carbon in plants and algae is ultimately derived from a single source, carbon dioxide, and they are called autotrophs. Nitrogen is obtained mostly as ammonia from bacterial metabolism of proteins from dead organism in the soil

2.1.2. The Light Reaction

Photosynthesis is the sunlight-mediated splitting of water into oxygen and energy. It occurs within a special membrane, the thylakoid membrane, which contains chlorophyll and surrounds a lumen. This membrane resembles the thick inner membrane of mitochondria or oxygen-utilizing bacteria. The thylakoid membrane lies within an organelle called a chloroplast, which is exclusive to leaf cells and algae where photosynthesis occurs. Central to the process of photosynthesis is the light-mediated loss of two electrons and two hydrogen atoms from a molecule of water (Fig. 2.2). Both electrons and one of the two hydrogen atoms pass to NADPH, the equivalent of NADH in mitochondria or bacteria; the other hydrogen atom becomes a hydrogen ion (H^+), a proton. When two molecules of water are split by chlorophyll, a molecule of oxygen (O_2) is released. For each molecule of oxygen, two molecules of NADPH and two protons (H^+) are made (Fig. 2.3).

Figure 2.4 illustrates electron and proton transport processes. Electrons are initially energized by sunlight hitting *photosystem II* (*PSII*; see figure legend) and transported to *photosystem I* (*PSI*). In *PSI*, sunlight energy is again imparted and the electrons are transferred by ferredoxin, another electron carrier, to NADPH. Electron transport from *PSII* to *PSI* is via *plastoquinone* (*PQ*), cytochrome b6/cytochrome f complex, and plastocyanin (blue arrows in Fig. 2.4). During electron transport, protons are taken up by plastoquinone (similar to

Fig. 2.2 In plants, light mediates the loss of two electrons and two hydrogen atoms from a molecule of water. See text for discussion of this process

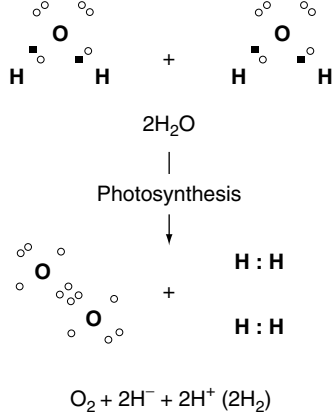
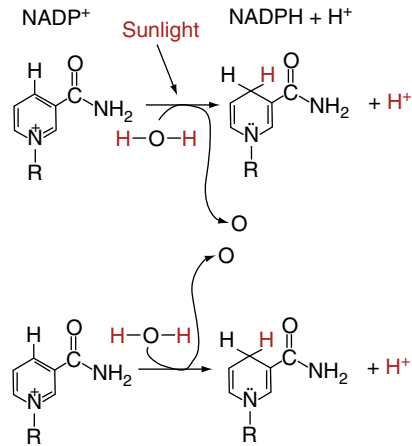


Fig. 2.3 The electrons are passed from water to NADPH. See text



ubiquinone in mitochondria) and released into the lumen (see legend to Fig. 2.4). As they accumulate, the protons start diffusing across the thylakoid membrane through an ATP synthase F_0/F_1 complex identical to that of mitochondria (red arrows in Fig. 2.4). Electrons that reach PSI are re-energized by sunlight and reduce NADP^+ by transport through ferredoxin (blue arrows in Fig. 2.4). The ATP, NADPH, and protons accumulate in the stroma of the chloroplast where they assist in the synthesis of triose phosphate and starch.

Figure 2.5 compares the orientation of the ATP synthase F_0/F_1 complex in mitochondria with that in chloroplasts. The lumen enclosed by the thylakoid membrane is slightly acidic; it corresponds to the mitochondrial intermembrane space where electron transport first pumps protons (H^+). In chloroplasts, ATP is made as protons diffuse from the thylakoid lumen through the membrane to the chloroplast stroma (Fig. 2.4). In mitochondria, ATP is made as protons diffuse from the mitochondrial intermembrane space through the inner mitochondrial membrane to the mitochondrial “lumen” or matrix.

**Photophosphorylation due to electron flow (blue)
and proton flow (red)
in chloroplast thylakoid membranes**

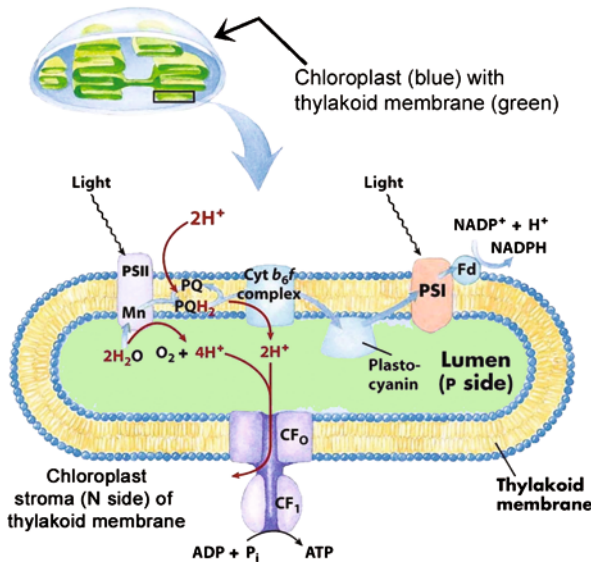
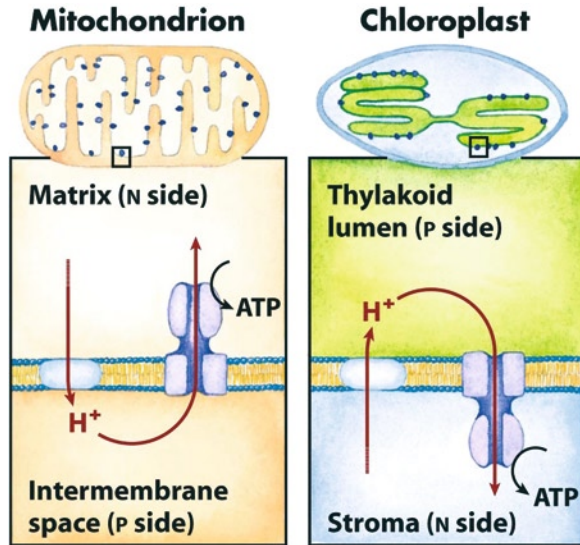


Fig. 2.4 Phosphorylation and electron transport in chloroplasts. Electron flow is shown in *blue* and the proton flow in *red*. The thylakoid membrane is colored *yellow* and the lumen is colored *green*. Light activates photosynthesis site II (*photosystem II* [*PSII*]) within chlorophyll whose manganese ions bind water molecules. Light rays attack a bound water molecule, breaking it up into oxygen atoms that combine to form oxygen gas, and hydrogen atoms that split into protons and electrons. Each two molecules of water give two pairs of electrons that are transported along with four protons to *plastoquinone* ($2PQH_2$). The protons are released into the thylakoid lumen (*red arrows*) as PQH_2 is reoxidized to PQ by the cytochrome complex (*blue arrows*). Protons accumulate in the lumen (P side of thylakoid membrane) and diffuse into the chloroplast stroma through carriers that synthesize ATP (CF_0 and CF_1 ; *purple* at the foot of figure). ATP is made from ADP and phosphate (P_i) in the chloroplast stroma (N side of thylakoid membrane). As detailed in the text, electrons are transported from PSII to photosystem I ([PSI], *pink*) where they are activated by light to reduce $NADP^+$ (*top right* of figure). Extra protons are taken back into the thylakoid lumen by plastoquinone. The plant partitions its electron flow and protons so that the ratio of ATP to NADPH matches its need for assimilation of carbon dioxide into carbohydrates, the primary product of photosynthesis (Adapted from Fig. 19-57 in Lehninger Principles of Biochemistry. D.L. Nelson & M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., New York)

Fig. 2.5 ATPase orientation in mitochondria and chloroplasts. *Blue dots* indicate the ATP synthetase in mitochondria (*top left*) and *green dots* in chloroplasts (*top right*). See text for further description (Adapted from Fig. 19-58 in Lehninger Principles of Biochemistry, D.L. Nelson & M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., New York)



Animals and bacteria are heterotrophs; they obtain carbon in various forms as food and metabolize many forms of it to provide energy and body structure. Plants are autotrophs; all their carbon comes from CO_2 powered by photosynthesis. Photosynthesis occurs within the thylakoid membranes of chloroplasts in plant leaves, and it is mediated by chlorophyll. The light reaction splits water into O_2 , electrons, and protons (H^+). NADPH is produced by electron transport and ATP synthesis by associated proton transport.

2.2.1. The Dark Reaction

The utilization of carbon dioxide by ATP and NADPH occurs in the chloroplast matrix, (outside the thylakoid lumen). A series of reactions assimilates carbon dioxide (Fig. 2.6), the Calvin cycle or dark reaction, and generates fructose 6-phosphate. Fructose 6-phosphate is the immediate precursor of glucose 6-phosphate for the synthesis of starch in the

Fig. 2.6 Overall design of photosynthesis. The light reactions make ATP and NADPH to be used in the carbon dioxide assimilation reactions that form fructose and glucose (Modified from Fig. 19-37 in Lehninger Principles of Biochemistry. D.L. Nelson & M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., New York)

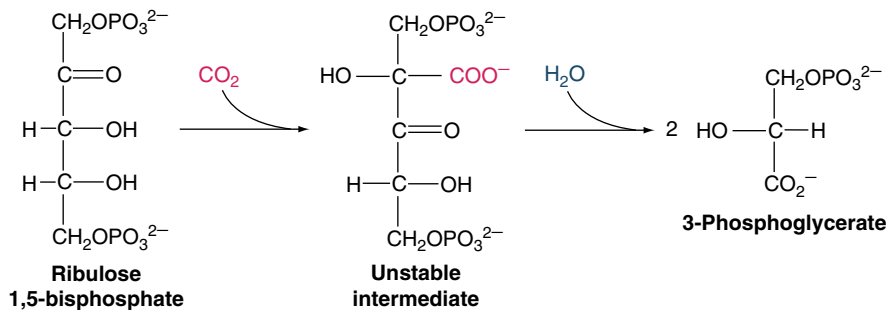
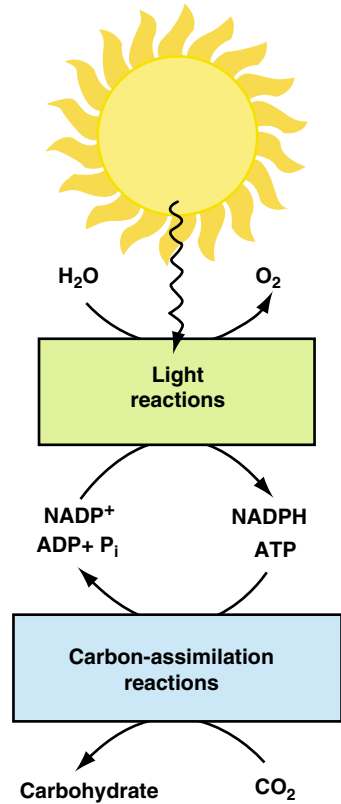


Fig. 2.7 Assimilation of carbon dioxide. The key reaction of the dark reaction is the assimilation of carbon dioxide by ribulose 1:5-bisphosphate carboxylase (Simplified from Fig. 26-31 in Biochemistry. L. Stryer, 4th Ed. 1995. W.H. Freeman & Co., New York)

chloroplast matrix and sucrose in the leaf cell cytosol. The substrate for carbon dioxide is ribulose 1:5-bisphosphate, and the reaction is mediated by a substrate-specific carboxylase, *rubisco* (Fig. 2.7). The products are two molecules of 3-phosphoglycerate, each of

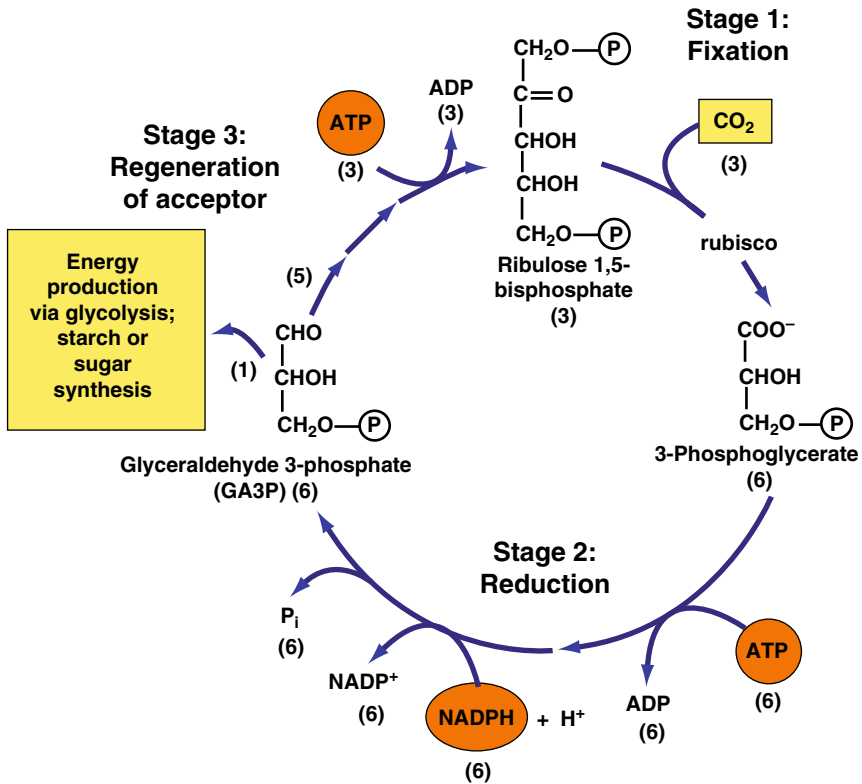


Fig. 2.8 Summary of the Calvin cycle. The cycle consists of three stages, culminating in the regeneration of ribulose 1:5-bisphosphate and a net increase in glyceraldehyde 3-phosphate. *Numbers in parenthesis* reveal the fate of carbon atoms entering and leaving the cycle (Modified from Fig. 20-04 in Lehninger Principles of Biochemistry. D.L. Nelson & M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., New York)

which reacts with an ATP molecule to make 1:3-bisphosphoglycerate, and then with NADPH to be reduced to glyceraldehyde 3-phosphate. For every six molecules of glyceraldehyde 3-phosphate obtained from three molecules of ribulose 1:3-bisphosphate, one is used for energy via glycolysis, or to synthesize monosaccharides or starch (Fig. 2.8). The other five interact to make three molecules of ribulose 5-phosphate, each of which utilizes three ATP molecules to regenerate three ribulose 1:5-bisphosphate molecules.

The enzymes that regenerate ribulose 5-phosphate from glyceraldehyde 3-phosphate are the same as those of the pentose phosphate path in non-photosynthetic organisms. The three stages of the Calvin cycle are (1) fixation of carbon dioxide by rubisco, which utilizes ATP; (2) reduction of the 1:3-bisphosphoglycerate to 3-bisglyceraldehyde, which utilizes ATP and NADPH; and, finally, the formation of ribulose 5-phosphate, which reacts with ATP to regenerate the rubisco substrate, ribulose 1:3-bisphosphate.

2.2.2.

Starch and Sucrose Provide the Carbon Skeletons of All Plant Compounds

During photosynthesis, starch is synthesized and stored in the chloroplast matrix and sucrose is synthesized in the leaf cytosol from which it diffuses to the rest of the plant. Starch resembles glycogen, but it has few or no α -1:6 glycosidic linkages, the glucose residues are little branched (amylopectin) or not branched (amylose). Amylose and amylopectin are substrates for salivary amylase, and their structures are illustrated in Fig. 12.10. The substrate for starch synthase is ADP-glucose, whereas the substrate for glycogen and sucrose synthases is UDP-glucose.

Sucrose is a highly soluble disaccharide that provides a mobile energy source for all the plant cells. Sugar cane stores large amounts of sucrose in its leaves and stalk, whereas sugar beet stores it in roots. *All plants make sucrose from two molecules of fructose 6-phosphate.* One molecule is activated with UDP and isomerized to UDP-glucose. Sucrose 6-phosphate synthase reacts with UDP-glucose and fructose 6-phosphate to make sucrose 6-phosphate. The latter then reacts with a phosphatase to produce sucrose (Fig. 2.9).

The amount of sucrose is regulated by a kinase that inhibits sucrose synthase by phosphorylating a serine residue on its polypeptide and a phosphatase that activates the synthase by dephosphorylating the serine residue. This mode of regulation resembles that of glycogen synthase in the mammalian liver. Both enzymes make enzyme energy storage compounds: sucrose for the plant as a whole and glycogen specifically for liver or muscles.

2.2.3.

Plants Are Autotrophs

The light-powered incorporation of carbon dioxide into ribulose biphosphate by the Calvin cycle enzymes synthesizes starch, which remains in the chloroplast stroma. Once the space for starch in the chloroplast stroma is exhausted, the 3-phosphoglycerate intermediate is converted by triosephosphate isomerase to *dihydroxyacetone phosphate (DHAP)*. DHAP is the only metabolite allowed to leave the chloroplast, and it does so in exchange for *phosphate (Pi)* from the cytosol (Fig. 2.10). The incoming Pi reacts with ADP to form ATP in the chloroplast, and it eventually ends up in a new molecule of triosephosphate. The exchange of DHAP for Pi is mediated by a protein transporter called an antiport that allows the two metabolites to pass in opposite directions through the membrane and down their respective concentration gradients. Similar antiport transport gradients are required for bone metabolism (Sects. 9.3.5, and 10.1.4). Once in the leaf cytosol, the DHAP is converted back to 3-phosphoglycerate, which is metabolized to various compounds, especially sucrose.

At night, there is no photosynthesis and respiratory energy becomes as important in plants as in other organisms. The starch that accumulated during the day is metabolized by the activation of phosphorylase and some glycolytic enzymes to triose phosphate (Fig. 1.7),

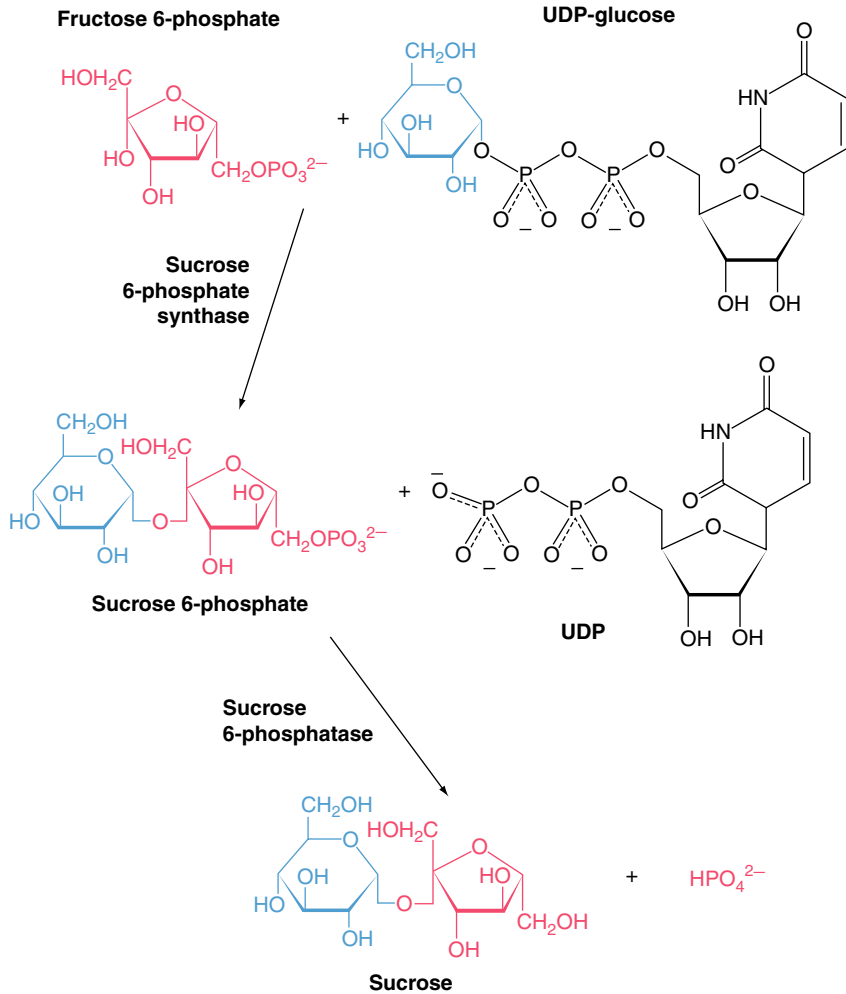


Fig. 2.9 Synthesis of sucrose. See text (Modified from Fig. 20-13 in Berg JM, Tymoczko JL and Stryer L. Biochemistry, 5th Ed. 2002. W.H. Freeman & Co., New York to also show the phosphatase step). Note: The diagram of fructose in this figure (*red*) is rotated so that its anomeric carbon atom (C2) lies to the right of the ring structure instead of the left as in a conventional diagram. Because of the rotation, it is not obvious that the fructose bond in sucrose is in the β -anomeric configuration. Only the glucose bond is in the α -anomeric configuration. The conventional diagrams of glucose and fructose alone and in sucrose are illustrated in Fig. 15.6.

which passes through the antiport as DHAP in exchange for P_i (Fig. 2.10). In the cytosol, DHAP moves to the mitochondria as a source of ATP. The P_i enters the chloroplast where it is a co-substrate for phosphorylase for starch breakdown.

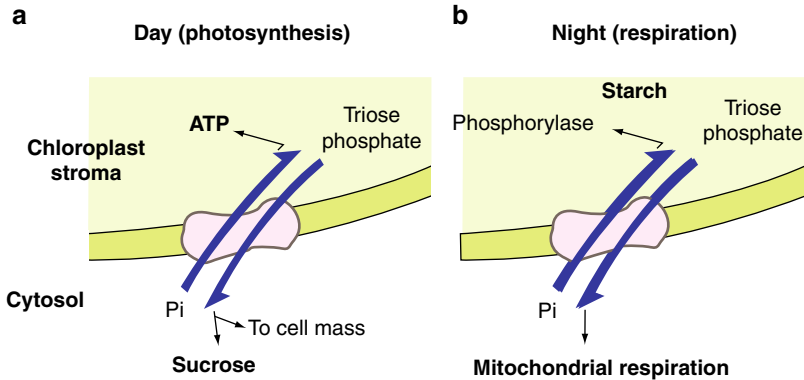


Fig. 2.10 Phosphoglycerate utilization. **(a)** *During the day.* Photosynthesis in the chloroplast makes starch until there is no more room. The Calvin cycle continues to make triose phosphate, which exits the chloroplast in exchange for organic phosphate (Pi) entering the chloroplast and converting ADP to ATP. In the cytosol, the triose phosphate is mostly converted to sucrose but also to small amounts of other compounds such as amino acids for transport throughout the plant. **(b)** *During the night.* Phosphorylase is activated and it breaks up the starch to glucose 6-phosphate from which triose phosphate is made. The triose phosphate is exchanged for Pi. The Pi is a substrate for phosphorylase and keeps it active. Once in the cytosol, the triose phosphate is transferred mostly to mitochondria for respiration

The dark reaction (Calvin cycle) uses the NADPH and ATP to make glyceraldehyde 3-phosphate (triose phosphate), which is metabolized initially to starch, sucrose, and cellulose. Starch and sucrose are the major plant storage products. Starch is synthesized from ADP-glucose in the chloroplast, sucrose from fructose 6-phosphate and UDP-glucose in the leaf cytosol.

2.3.1.

Sucrose Is the Primary Transport Sugar and Plays a Central Role in Plant Growth and Development

The availability of metabolites for sucrose synthesis and the need for products of sucrose degradation regulate gene expression. For respiration, sucrose is hydrolyzed by *invertase* to free glucose and fructose, which are phosphorylated and undergo glycolysis to pyruvate. The pyruvate is then either metabolized by mitochondrial electron transport to ATP and NADH (respiration), or metabolized to provide starting products for amino acid, lipid, and nucleotide syntheses.

Cellulose helps protect plants from their environment and to access sunlight by providing a firm structure. Cellulose is $\beta 1 \rightarrow 4$ glucose, whereas starch is $\alpha 1 \rightarrow 4$ glucose. Both are derived from sucrose outside of chloroplasts. The sucrose synthase reaction (Fig. 2.9) is reversed: sucrose is phosphorylated with ATP to make sucrose 6-phosphate, an excess of which reverses the reaction. The products are UDP-glucose and fructose 6-phosphate. Cellulose is synthesized from UDP-glucose by cellulose synthase. Additional UDP-glucose reacts with ATP to form ADP-glucose, the precursor of starch. The fructose 6-phosphate is metabolized to provide the necessary energy (ATP and NADPH) by respiration.

Sucrose plays a central role in plant growth and development. Invertase-catalyzed hydrolysis of sucrose is associated with the respiration required for plant growth, whereas sucrose synthase-catalyzed hydrolysis is linked to cell wall or other storage product biosynthesis. Cellulose is synthesized from UDP-glucose using cellulose synthase in membranes. Cellulose protects plant cells from the environment and provides them with a firm structure to access sunlight.

Dentists are concerned with the structures of the oral cavity, the teeth, the oral mucosa, the underlying dermis and the alveolar bone. The dermis beneath the oral mucosa and surrounding the teeth is similar to the dermis beneath the skin. Both are primarily composed of collagen fibers within a connective tissue or stromal matrix. Variants of this matrix are present in the soft and hard tissues of the body, including the teeth, gingiva, periodontium, and alveolar bone. The Sects. 1 and 2 of this introductory chapter describe the gingival stromal matrix and its major components. Sect. 3 describes how the cells and stroma interact. Subsequent chapters describe in detail the structures of the various types of collagen, elastin, and other proteins and proteoglycans that contribute to the matrix (Chap. 6), the synthesis and breakdown of collagen (Chaps. 7 and 8), the structure and synthesis of bone and tooth enamel (Chap. 9), and bone dissolution and turnover related to overall calcium metabolism (Chap. 10).

3.1.1.

Major Components of the Connective Tissue (Stromal) Matrix

The *stroma* or internal solid mass of an organism comprises its cells and embedded matrix. The major components of the *stromal matrix* of vertebrates are collagen fibers embedded within a polysaccharide ground substance of hyaluronan and various proteo-glycosaminoglycans (Fig. 3.1). The *stromal cells* are derived from mesodermal lineage cells that have differentiated into fibroblasts, chondroblasts, and osteoblasts that make, respectively, connective tissue, cartilage and bone. The stroma and bone are permeated with low molecular weight nutrients (glucose, amino acids, vitamins, etc.) and oxygen. Within the blood, some white cells become attracted to the stroma and differentiate, depending on the tissue and its environment. The stromal matrix is covered by an *epithelium* derived from ectoderm (e.g., epidermis or oral mucosa), or endoderm (e.g., capillary, lung alveoli, kidney tubules, or gut lining). The stroma beneath an epidermis is referred to as the dermis.

Where a stromal surface meets the epithelium, the fibroblasts interact with epithelial cells to form a *basal lamina*. This thin, flexible layer of specialized extracellular matrix (40 to 120 nm thick) underlies all epithelial cell sheets or tubes and is detected by

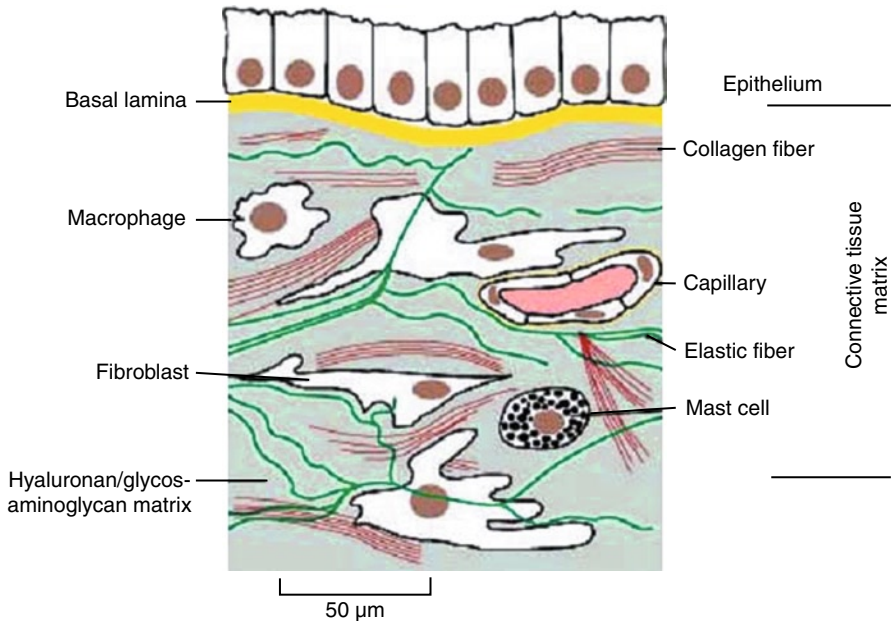


Fig. 3.1 Composition of the connective tissue extracellular matrix. Collagen fibers, maroon; Elastic fibers, green; Hyaluronan-proteoglycan matrix, gray. Fibroblasts, a macrophage, a mast cell, and a capillary containing a red blood cell are also shown (Modified from Fig. 19–34 in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, NY)

visualizing the tissue under an electron microscope. Figure 3.2 shows three alternative organizations of a basal lamina: surrounding skeletal muscles, underlying epithelia, and interposed between two cell sheets as in the kidney glomeruli. A basal lamina acts as a semipermeable membrane, to separate specialized cells such as epithelial or muscle cells from soluble stromal proteins, or in the case of the kidneys, to act as a membrane through which metabolic end products from the body can pass. The rich capillary network immediately beneath an epithelium ensures that essential nutrients and low molecular weight cell-modifying agents (chemokines) have access to the overlying cells. Basal laminas prevent soluble plasma proteins from passing into stromal fluid or soluble stromal proteins from passing into epithelial fluid.

3.1.2. Collagen

Collagen is the major protein of the stroma. There are two major groups of collagens encoded in the genome: fibrillar and non-fibrillar (Table 3.1). Collagen *fibers* are the most abundant group and visible to the naked eye. Collagen *fibrils* are visible only through a

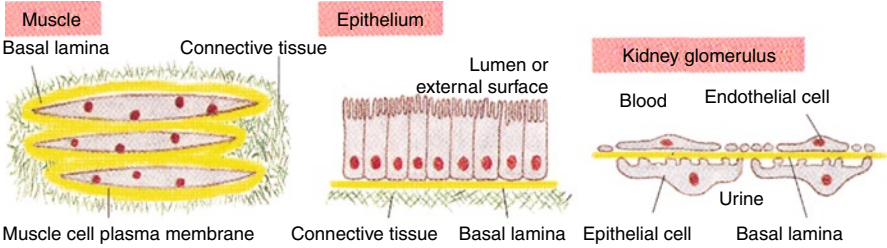


Fig. 3.2 Relationship of the connective tissue stroma to an epithelium. Fibroblasts secrete unique types of laminin proteins that interact with each other and type IV collagen, to form a basal lamina (yellow) that is tightly attached to the connective tissue (stromal) cells and also to the associated muscle or epithelial cell. The kidney glomerulus is a specialized tissue in which the stroma is absent and endothelial cells and epithelial cells are separated by the basal lamina which filters the blood as the first step in urine collection. The attachment of an epithelium to stromal collagen is discussed in detail in Chap. 5 (Fig. 19-55 in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, NY)

Table 3.1 Major functions, genetic, and polypeptide composition of some common types of collagen

Collagen functions and types	Gene name	Polypeptide composition
FIBRILLAR collagens (Types I, II, III, V, & XI)	COL1A1, COL1A2	$[\alpha_1(\text{I})]_2[\alpha_2(\text{I})]$ (Most common)
	COL2A1	$[\alpha_1(\text{II})]_3$
Dermal reticular fibers (Type III with V)	COL3A1	$[\alpha_1(\text{III})]_3$
	COL5A1, COL5A2 COL11A1, COL11A2	$[\alpha_1(\text{V})]_2[\alpha_2(\text{V})]$ $[\alpha_1(\text{XI})]_2[\alpha_2(\text{XI})]$
NETWORK (fine bone marrow/ epiphyseal reticular fibers) (Type III with VIII & X)	COL8A1, COL8A2	$[\alpha_1(\text{VIII})]_2[\alpha_2(\text{IV})]$
	COL10A1	$[\alpha_1(\text{X})]_3$
BASEMENT MEMBRANE collagens (Type IV)	COL4A1, COL4A2,	$[\alpha_1(\text{IV})]_2[\alpha_2(\text{IV})]$
	COL4A3	(In all tissues)
	COL4A4, COL4A5,	$[\alpha_3(\text{IV})][\alpha_4(\text{IV})][\alpha_5(\text{IV})]$
	COL4A6	(Glomerulus) $[\alpha_5(\text{IV})]_2[\alpha_6(\text{IV})]$ (Restricted)
MICROFIBRILLAR collagens (Type VI)	COL6A1, COL6A2, COL6A3	$[\alpha_1(\text{VI})][\alpha_2(\text{VI})][\alpha_3(\text{VI})]$
ANCHORING fibrils with interrupted triple helix; long chain collagen (Type VII & XVII)	COL7A1, COL17A1	$[\alpha_1(\text{VII})]_3 [\alpha_1(\text{XVII})]_3$
FACIT Fibril-associated collagens with interrupted triple helices (Types IX & XII)	COL9A1, COL9A2,	$[\alpha_1(\text{IX})][\alpha_2(\text{IX})][\alpha_3(\text{IX})]$
	COL9A3 COL12A1	$[\alpha_1(\text{XII})]_3$

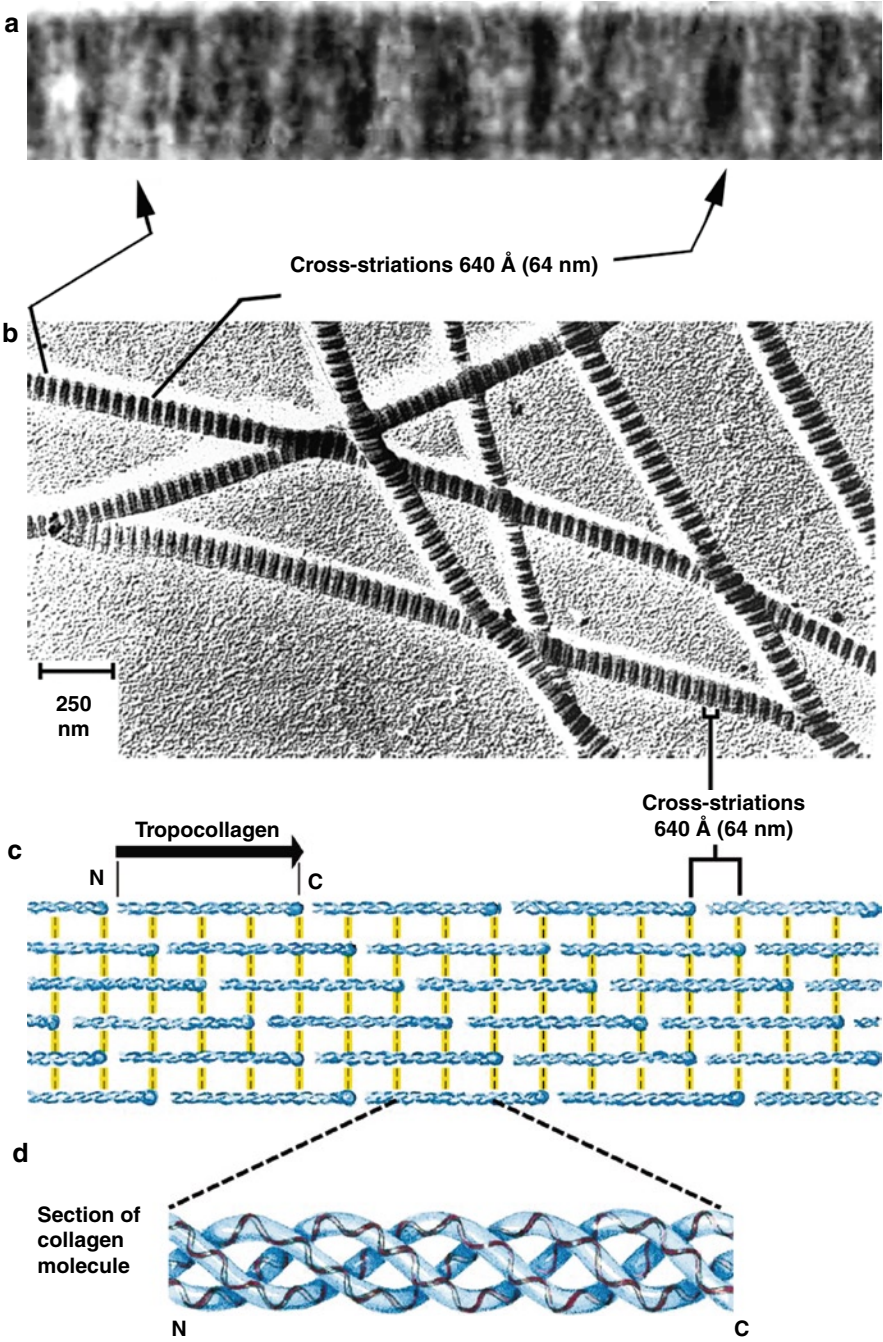
Modified from public domain data at <http://en.wikipedia.org/wiki/Collagen>

light microscope and collagen *microfibrils* or *filaments* only though an electron microscope. In addition to forming and maintaining tissue integrity and stability, collagens also form a bioactive surface that regulates cell differentiation, morphogenesis, and migration, as well as, wound repair, inflammation, and thrombosis. In addition, alterations in collagen metabolism lead to a large spectrum of diseases, including common disorders like osteoarthritis and atherosclerosis. An equal number of other genes encode collagen-like domains in various other proteins such as the complement proteins of blood plasma (see Sect. 3.3.2.).

The fibrillar collagens are made from types I, II, III, V, and XI polypeptides (Table 3.1). The most predominant collagen is type I, but mixtures with other collagen types affects the fiber structure. Type I fibers are often found as a complex with type V fibers for various reasons: to facilitate corneal transparency; to limit fiber thickness during tissue repair and to help form the architecture of various collagen-containing tissues such as tendons or the placenta. Type II fibers, which are unique to cartilage, form a complex with type XI collagen to limit thickness and enhance binding to proteo-glycosaminoglycans. Reticular fibers are delicate fibers composed mainly of type III collagen and extensively covered with glycosaminoglycans and glycoproteins. In the dermis of the skin and gingiva, the reticular fibers extend out from type I collagen fibers that have already associated with type V polypeptides. Within the bone marrow and other less fibrous tissues, the type III collagen of reticular fibers associates with a mixture of non-fibrillar type VIII and type X polypeptides.

All collagen fibers, fibrils and microfibrils have an alternating light and dark staining pattern (striated or banding pattern) that has a characteristic appearance (upper half of Fig. 3.3). This pattern is due to the staggered array of tropocollagen triple helices (lower half of Fig. 3.3, also discussed in Sects. 4.2.1, 8.2.1 and 8.3.4). The fibrillar collagens are synthesized as a precursor, procollagen. On secretion, the *N*-terminal and *C*-terminal domains are removed. Sequences identical or similar (*homologous*) to the *N*-terminal procollagen domain of the commonly found fibrillar collagens are expressed on other proteins and are referred to as procollagen domains.

Fig. 3.3 The striated appearance of collagen fibers. Collagen fibrils are made up of tropocollagen molecules aligned in a staggered fashion and cross-linked for strength. **(a)** Electron micrograph showing cross-striations from native rat tail collagen. Alternate, broad, mainly light and mainly dark bands with a period of approximately 64 nm are prominent (From Fig. 1 of Cox RW, Grant RA and Kent CM (1972) "The interpretation of electron micrographs of negatively stained native collagen." *Journal of Cell Science* 10:547–554). **(b)** Less magnified view of collagen fibers. **(c)** Self-aggregation of tropocollagen. The *N*- and *C*-terminal ends of tropocollagen are called telopeptide domains and they interact with adjacent tropocollagen molecules (*yellow lines*). **(d)** Tropocollagen self-aggregating unit. A tropocollagen molecule is a self-aggregating unit of three long polypeptides that are intertwined in a triple helix. It is cut out from a genetically encoded polypeptide called procollagen as described in Sect. 4.2.1 (Figs. **(b)**–**(d)**) modified from Fig. 4–13 in Lehninger Principles of Biochemistry. D.L. Nelson and M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., NY)



The kidney contains small amounts of fibrillar collagen, but its major collagen is a non-fibrillar network collagen (type IV), a part of basal laminas called the lamina densa (Sect. 5.1.1). Basal laminas are especially well developed in the kidney as exemplified in the glomerulus (Fig. 3.2). The collagen contents of various tissues are indicated in Table 3.2 and their fiber arrangements in Table 3.3.

3.1.3.
Elastic Fiber System

Elastic fibers endow a stroma with recoil after stretching. They are composed of *fibrillin* with or without a central portion of *elastin* (Table 3.4). These two proteins are described in detail in Sect. 6.1.1 and 6.2.1). *Elastic fibers* are especially prominent in *ligaments* and

Table 3.2 Collagen and elastin content of some tissues (g/100 g of dry weight)

Tissue	Collagen	Elastin
Ligament	17	50
Aorta	12–24	30–57
Tendon (achilles)	86	4
Dermis of skin	72	2–5
Liver	3.9	0
Cartilage	46–63	0
Cornea	68.1	0
Bone, mineral free	88	0

Collagen data is from Table 2.9 in R. Montgomery et al. *Biochemistry, A case oriented approach*, 4th Ed., The CV Mosby Co., St Louis MO 1983. Elastin data is from S. Mithieux and A.S. Weiss, *Adv. Prot. Chem.* 79:437–461, 2005

Table 3.3 Collagen fiber arrangements in some tissues

Tissue	Arrangement of fibrils or microfibrils ¹
Tendon/Periodontium	Parallel fiber bundles
Cartilage	No distinct arrangement
Dermis of skin	Planar sheets of microfibrils layered at many angles
Cornea	Planar sheets stacked crossways for strength

¹Fibers seen by naked eye, fibrils only by light microscope, microfibrils only by electron microscope

Data is from Table 2.10 in R. Montgomery et al. *Biochemistry, A case oriented approach*, 4th Ed., The CV Mosby Co., St Louis, MO, 1983

major arteries (Table 3.2). Thin elastic fibers consist of fibrillin bundles around very little elastin (*elaunin fibers*). Within the dermis of the skin there is a core of thick elastin fibers within which a deeply embedded elaunin plexus radiates thin fibers towards the superficial layers beneath the epidermis. *Oxytalan fibers* are fibrillin bundles with no elastin. They are important at four major sites throughout the body (Fig. 3.4): (A) Between the fiber bundles of the periodontium (where they were first found and described below, Sect. 3.1.5); (B) beneath the endothelial cell lining of blood vessels, also called the intima (Sect. 11.1.1); (C) in the dermis perpendicular to the dermo-epidermal junction; and (D) beneath the epithelial covering of organs or tissues (adventitia) such as surrounding lung alveoli (Tables 3.2 and 3.4).

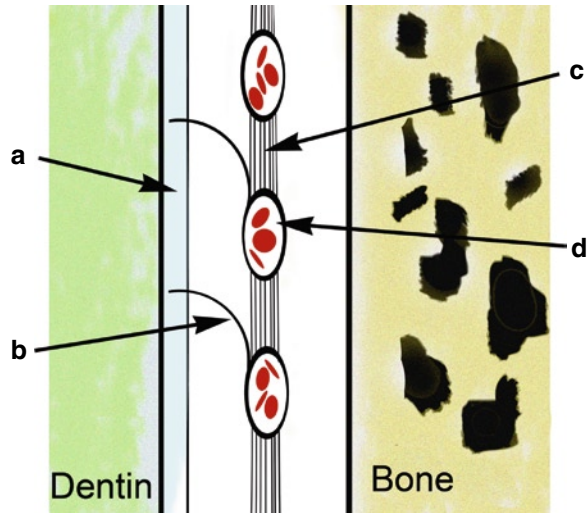
Unlike *ligaments* which attach bone to other bones or teeth, *tendons* attach bone to muscle and have few elastic fibers (Table 3.2). Ultraviolet radiation and aging disorganize the elastic fiber system of the skin by activating proteases that degrade elastin and fibrillin.

Table 3.4 Composition of three major types of elastic fibers

Fiber type	Arrangement	Tissue ^a
Elastic	Elastin surrounded by fibrillin	Ligaments & large arteries
Elaunin	Fibrillin with a little elastin	Deeper layer of skin dermis
Oxytalan	Fibrillin only	Throughout body

^aMore details in text

Fig. 3.4 An oxytalan fiber tract. (a) Cementum; (b) Principal oxytalan fiber; (c) Oxytalan tract; (d) Periodontal vessel (From Fig. 2.4, Oxytalan fibers forming a network that attaches blood vessels to the cementum. In article titled, “Force Generation and Reaction within the Periodontium.” Caputo AA and Wylie RS: UCLA School of Dentistry website; <http://www.dent.ucla.edu/pic/members/force/index.html>) Figure modified slightly and redrawn by Dr Wirsig-Weichmann. Permission granted by the Authors



Elastin is efficiently synthesized only in the fetus and children because it is normally extremely stable (half life ~70 years). By contrast fibrillar collagens including those in bone have a turnover half-life of about 6 months. The failure to replace elastin causes the skin to become less elastic, more wrinkled, and thinner with age.

3.1.4.

Glycosaminoglycans

Glycosaminoglycans are carbohydrates important for the development and repair of the stroma, and also for the formation of cartilage. During an organism's development, or following the removal of damaged tissue from an infection or injury, fibroblasts are activated to proliferate and secrete hyaluronan, a large, repeating dimer of glucuronate linked to *N*-acetylglucosamine. The fibroblasts then invade the hyaluronan (ground substance) along with proliferating endothelial cells that form new capillaries. Once within hyaluronan, the fibroblasts secrete collagen fibers that increase stromal density. Proteo-glycosaminoglycans are proteins that have covalently bound glycosaminoglycans. They are synthesized by fibroblasts, chondroblasts, and osteoblasts along with collagen. These negatively charged polymers impart resilience (pliability) to the stroma, and are especially important in cartilage. The stroma is both resilient and fibrous. Long hyaluronan molecules are especially important in maintaining the viscosity of joint fluid around the cartilage between bones, and within the eyeballs. Fibroblast production of shorter length hyaluronans stimulates angiogenesis (new capillary formation).

3.1.5.

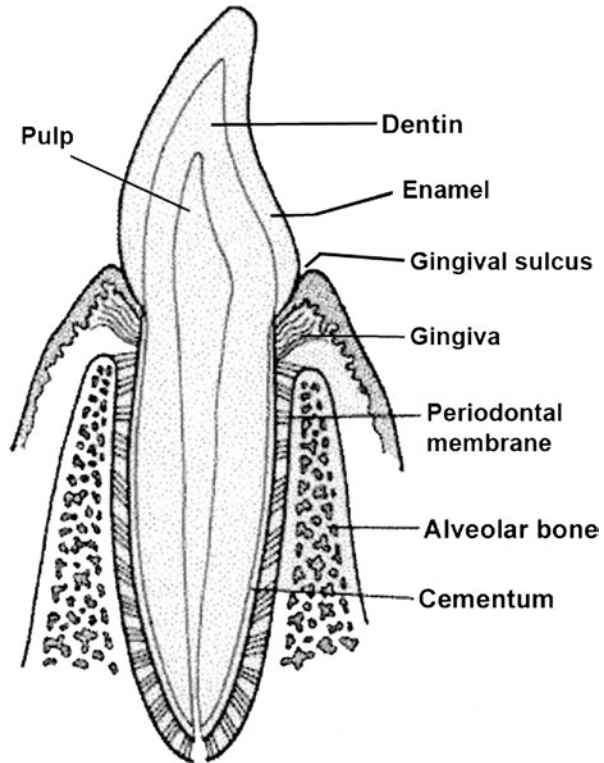
Alveolar Bone, Teeth, and Periodontium

Bone is synthesized by osteoblasts which transport calcium ions from blood into a secreted, uncalcified osteoid matrix composed mostly of type I collagen. During calcification, monocyte-like cells are attracted out of the adjacent blood capillaries and adhere to irregularities in the calcifying bone surface and eventually become osteoclasts. These cells, which resorb the bone, develop according to genetic and environmental stimuli that determine bone shape and response to stress.

A tooth and its surrounding tissue (the *periodontium*) are diagrammed in Fig. 3.5. The enamel is made by ameloblasts, cells of ectodermal origin unrelated to osteoclasts which are of mesodermal origin. Enamel is the only calcified tissue that does not contain collagen in the vertebrate body. It forms a tight, impenetrable seal around the dentin, much like a skin. The dentin and cementum are calcified over type I collagen fibers like bone. They are respectively made by odontoblasts and cementoblasts, both closely related to osteoblasts. Once dentin has been synthesized, odontoblasts remain viable on its inner surface

Fig. 3.5 The periodontium.

The periodontium consists of the gingiva and underlying periodontal membrane. Both are composed of large bundles of collagen mixed with large and small oxytalan fibers that allow the periodontium to respond to violent movements of the tooth within the jaws during mastication



within the pulp cavity, a region of uncalcified connective tissue rich in blood vessels and nerves in the center of the teeth (Fig. 3.5). On the outer surface of dentin apical to enamel, cementoblasts differentiate from fibroblasts and form *cementum*. The synthesis of the calcified tissues is discussed in detail in Chap. 9.

The periodontium comprises the *gingiva*, *cementum*, *periodontal ligament*, and surrounding alveolar bone. The gingiva and periodontal attachment are largely composed of large collagen fiber bundles to absorb masticatory forces. The periodontal collagen fibers are called *periodontal ligaments* because both ends are calcified like the ligaments that attach bones. Instead of elastin, the ligaments and blood vessels are surrounded by oxytalan fibers composed of fibrillin which provide the lesser elasticity required for tooth movement as compared with bone movement. The gingiva lies coronal to the periodontal ligaments and is composed of free and attached segments. The free gingiva is the soft tissue wall adjacent to the gingival sulcus. Its coronal end is the gingival margin and its apical end is continuous with the attached gingiva on the oral side and with a *junctional epithelial attachment* on the dental side (Fig. 3.6). The free gingiva is covered by keratinized epithelium on its oral cavity surface and non-keratinized epithelium in the sulcus against the tooth surface (Sects. 5.2.2 and 5.2.3).

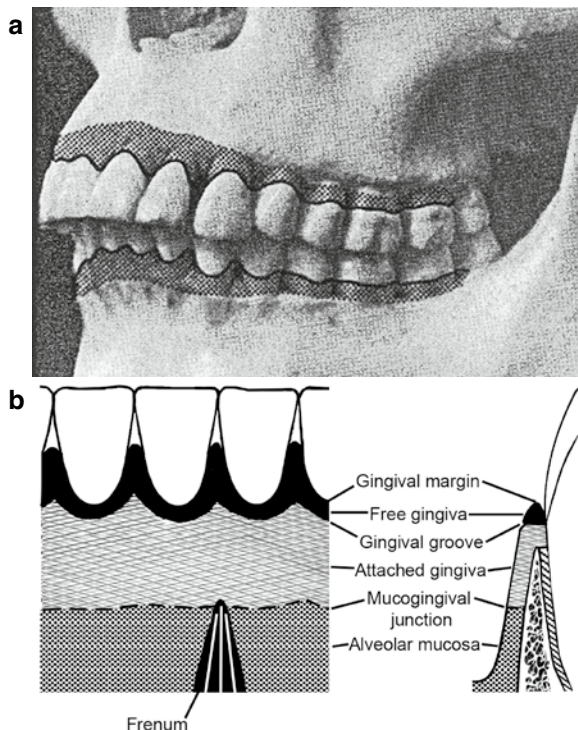


Fig. 3.6 Diagrams showing the attached gingiva (cross-hatched) and free gingiva (dark line against the teeth): (a) is a clinical view and (b), enlarged horizontal and cross-sectional views. The coronal end of the *attached gingiva* is the *free gingiva* which lies beneath the *gingival margin* and extends apically for 1 – 2 mm to the gingival groove where collagen fibers attach the gingival epithelium to alveolar bone. The free gingiva forms the outer wall of the *gingival sulcus*. The free gingiva is shorter and the attached gingiva longer than in this diagram which is not drawn to scale. In periodontitis (Chap. 13), the gingival margin migrates apically to beneath the cemento-enamel junction, causing the attached gingiva to shorten in height and the cementum of the teeth to appear in the oral cavity. (From Fig. 1–1, *Contemporary Periodontics*, edited by RJ Genco, HM Goldman and DW. Cohen. Chapter 1: The Gingiva, Structure and Function by H Loe., MA Listgarten & VP Terranova. Publisher was The CV Mosby Co., St Louis, MO, 1990; Copyright Elsevier, 2008). Figure was modified slightly and redrawn by Dr Wirsig-Weichmann

The gingiva and periodontium both possess five groups of collagen fibers or ligaments, which are categorized by orientation and function. The gingival fibers are: (A) dentogingival; (B) alveologingival; (C) circumferential; (D) transseptal; and (E) periosteal (Fig. 3.7). The first two are free gingival fibers, which are calcified into cementum or bone at one end and free at the other. They hold the free gingiva tightly against the tooth surface. The gingival circumferential fibers anchor in the cementum and partially encircle a tooth beneath the free gingiva. The transseptal fibers are

ligament-like fibers attached to cementum at each end. They maintain teeth alignment by connecting the teeth interdentally (not shown in Fig. 3.7). Periosteal fibers hold the attached gingiva tightly to the outer surface of alveolar bone (Fig. 3.7). The periodontal ligament fibers are also called the principal fiber bundles based also on location and orientation: (A) alveolar crest; (B) horizontal; (C) oblique; (D) periapical; and (E) interdental in multi-rooted teeth. The portions of these fibers anchored into cementum or alveolar bone are called *Sharpey's fibers*.

The stroma within which the principal periodontal fibers are embedded is composed of proteoglycans within which are embedded oxytalan fibers (Sect. 3.1.3) whose elasticity is important (Sect. 3.1.3). Mini-oxytalan fibers surround blood vessels and are attached to

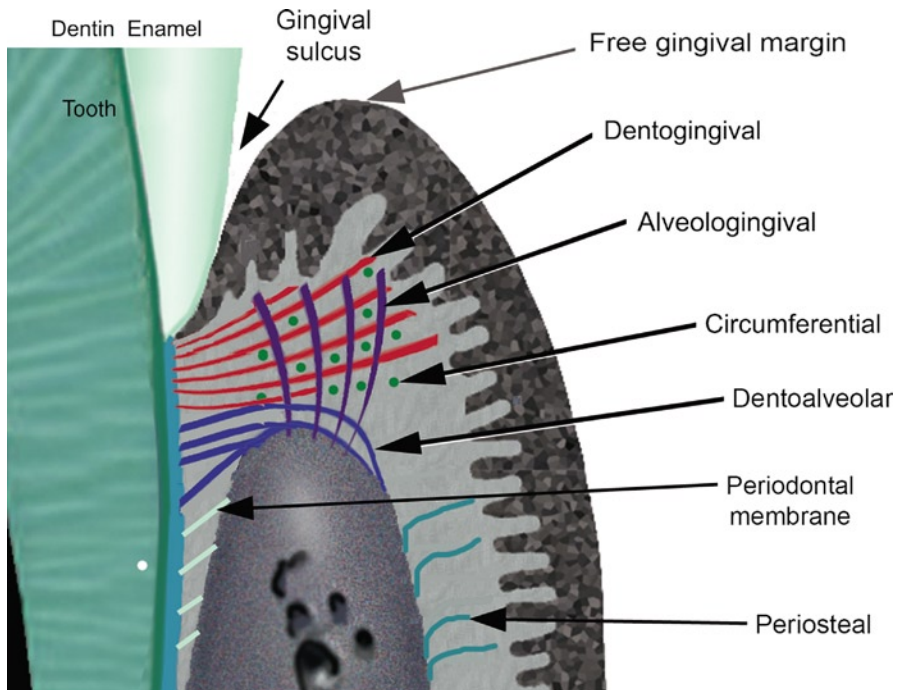


Fig. 3.7 Gingival fiber groups. Two of the four groups of gingival collagen fibers are indicated: dentogingival and alveologingival. The third group, the circumferential fibers, is shown as green circles in vertical cross section. The fourth group, the transseptal fibers, is not shown. These fibers run between the teeth and are obscured by the teeth in the plane of the figure. The dentoalveolar fibers lie immediately above the periodontal membrane fibers. The periosteal fibers hold the attached gingiva tightly to the alveolar bone and are not part of the periodontium. The fibers are briefly described in the text (Modified from a PowerPoint slide entitled “Dental, Gingival Fiber Groups” accompanying an Histology Full-Text: William A Beresford: West Virginia University. <http://wberesford.hsc.wvu.edu/histolch24.htm>). Figure modified slightly and redrawn by Dr Wirsig-Weichmann. Permission given by author

cementum by regular oxytalan fibers (Fig. 3.4). These fibers may support the blood vessels within the periodontium during chewing. Biting causes the teeth to compress the blood vessels in the periodontium. Oxytalan fibers allow the capillaries to recoil so that the blood supply with its oxygen and other essential nutrients is immediately replaced when the pressure is released in the human periodontium.

The connective tissue extracellular matrix along with the associated cells comprises the stroma or internal solid mass of an organism. Collagen is the major stromal protein in vertebrates. It may be fibrillar or non-fibrillar, depending on the types of collagen polypeptides expressed. Fibrous collagen forms filaments, microfibrils and fibers in ascending order of size and molecular complexity. Other fibers in the stroma are made from fibrillin and elastin. The various stromal fibers provide tensile strength, filtration barriers, and shape to the tissue or organ. The stroma is separated from the overlying epithelial cells by a basal lamina. The basal lamina acts as a filter to control the movement of proteins and other molecules between fluid compartments, e.g., between the blood and stroma or between the stroma and epithelium. Collagen interacts with other proteins, glycans and proteoglycans to build calcified and uncalcified tissues and organs. Bone, dentin, and cementum are formed from collagen fibers synthesized by osteoblasts, odontoblasts, and cementoblasts, respectively, and around which calcium phosphate has precipitated. Uncalcified collagen fiber bundles, and collagen calcified in cementum and bone are the major components of the gingiva and periodontium. The ends of the collagen fibers of the periodontal ligament are embedded in calcified cementum and bone to anchor the tooth into the bony socket. Collagen fibers in the gingiva provide structural support to the gingival tissue and maintain the alignment of the teeth. Oxytalan fibers are made of fibrillin and elastin. They support the blood vessels within the periodontium during chewing.

3.2.1.

Cell Surface Binding: Integrins, Fibronectin, and Collagen

The stromal fibers generally remain tightly attached to fibroblast, chondroblast, or osteoblast cells that produce them. The fibroblast cell surface contains *integrins*, a family of proteins that attach laminin, fibronectin and many other stromal proteins by an Arg-Gly-Asp motif (RGD in the one-letter amino acid code; see also Sects. 4.4.1, 5.1.2, 8.2.1, 10.1.1, and 11.2.1). An important insight into the importance of the RGD motif of fibronectin and other proteins in mediating stromal cohesion came from snake bites. A family of polypeptides (45–84 amino acids) in snake venoms, the modified saliva of many snakes, contains the RGD sequence. These polypeptides are known as *disintegrins*. They break up or disintegrate the stroma by competing out normal integrin–ligand interactions including platelet aggregations

that normally initiate blood clots (Sect. 11.2.1) at the site of a snake bite. The disintegrin activity permits other components of snake venom such as nerve inhibitor enzymes to reach the systemic circulation.

Collagen binds integrins by an unrelated motif, a short, proline-poor peptide which is flexible within the triple helix backbone as discussed in Sect. 4.4.1. Fibronectin is a complex multidomain protein containing a motif that recognizes integrins (FN-1 motif) and collagen fibers (FN-2). A third fibronectin motif (FN-3) recognizes heparin sulfate, a cell surface glycosaminoglycan (Sect. 6.3.1). Fibronectin usually forms a dimer that exposes the integrin-binding RGD motif in one region and the collagen binding motif in another. Thus, fibronectin attaches collagen fibers in the stroma to cell surface integrins independently of collagen-integrin binding. This separation allows collagen alone to control the cell cycle and its own synthesis (Sect. 4.4.1). The segment of the triple helical region of collagen that binds to the fibronectin FN2 motif is rich in proline and entirely separate from the collagen integrin-binding motif.

3.2.2. Thrombospondins and Transforming Growth Factor- β

Thrombospondins are five independently encoded proteins unique to vertebrates. The original *Thrombospondin* (TSP-1) was first identified as a product of thrombin-activated platelets during blood clotting (Sect. 11.3.4), and was designated a “thrombin-sensitive protein.” The TSP protein family is divided into two groups that differ in structure and biological role (Fig. 3.8). Group A consists of TSP1 and TSP2, which are homotrimers of a 145 kDa polypeptide. Human thrombospondin-1 is secreted as a 1152 amino acid chain following loss of an 18 amino acid secretion signal. Group B (TSP3, TSP4 and TSP5) are homopentamers, but their subunit polypeptide is smaller (~110 kDa corresponding to 934 amino acids after removal of a 22 amino acid *N*-terminal signal sequence). The group B family lacks a procollagen domain (see legend to Fig. 3.8) and repeats of the type I proteins (TSB type I repeats), but it contains four rather than three type II TSB repeats which are related to epidermal growth factor protein.

TSP-1 functions by activating *transforming growth factor- β* (TGF- β), a 112 amino acid protein whose three variants (β 1, β 2, and β 3) control proliferation, differentiation, apoptosis (Sect. 13.4.1), and various other functions in many different cell types. All three forms of TGF- β have three domains: (1) an *N-terminal signal peptide* of 20–30 amino acids that is required for secretion and removed in the endoplasmic reticulum; (2) an intermediate 280 amino acid *pro-region* (called the latency associated peptide or LAP); and (3) a 112–114 amino acid *C-terminal region* that becomes the mature TGF- β molecule. TSP-1 binds to LAP, and this exposes the *C*-terminal domain to cleavage by stromal proteases and forming the active TGF- β .

The active portion of TGF- β (the *C*-terminal region) is composed of a *cysteine knot*, nine conserved cysteine residues, of which eight form disulfide bonds within the molecule

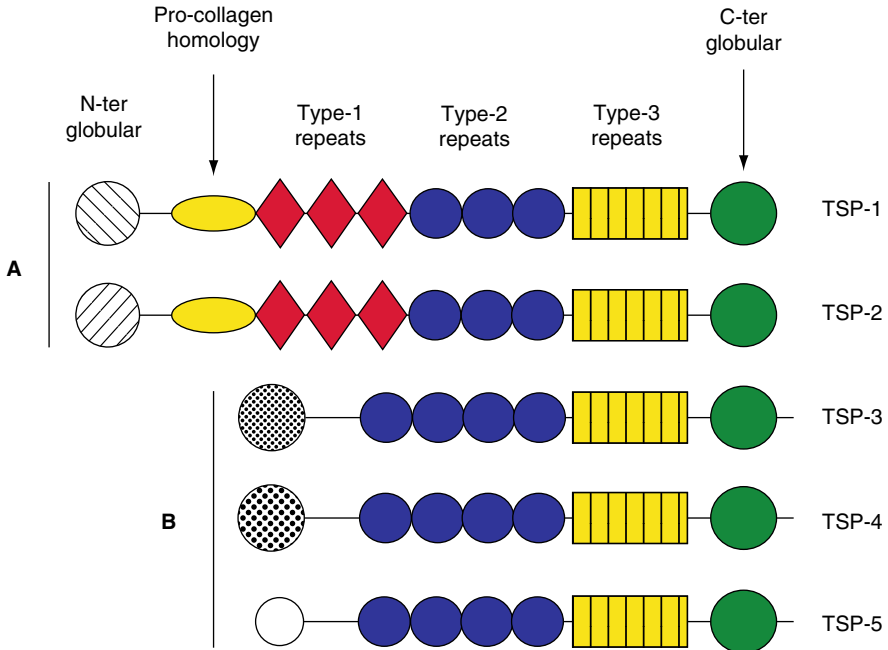


Fig. 3.8 Domain organization of the thrombospondin gene family. Shapes and colors illustrate the different domains discussed in the text. The procollagen domain is homologous to C- and N-terminal domains that are excised from collagen polypeptides when a fiber forms (Modified from Fig. 1 in Adams JC, Tucker RP (2000 Jun) “The thrombospondin type 1 repeat (TSR) superfamily: diverse proteins with related roles in neuronal development.” *Developmental Dynamics*, 218(2):280–299)

to create a TGF- β superfamily structure, while the ninth cysteine connects to the ninth cysteine of another TGF- β molecule to produce the active form, a dimer. The free cysteine residues of an almost identical cysteine knot sequence at the C-terminus of von Willebrand factor (Sect. 11.2.1) similarly causes dimer formation. A virtually identical structure at the C-terminus of *salivary Mucin Glycoprotein-1* (MG-1; Sect. 12.3.1) is partially responsible for its multi-monomeric structure.

Many cells secrete at least one of the three immature forms of TGF- β , and essentially all cells have receptors that respond to the presence of mature TGF- β in the stroma. In the periodontium, TGF- β stimulates fibroblast and osteoblast proliferation during connective tissue or bone remodeling (Sect. 10.1.3), and maintains the proliferation of dentally attached epithelial cells (Sect. 5.2.3). The linker domains that connect calcium binding domains in fibrillin are identical to the sequence of protein receptors that bind to TGF- β (Sect. 6.1.1).

Thrombospondin-2 is the major expressed thrombospondin in the human body. Mice deficient in TSP2 exhibit excessive collagen degradation. Secretion of TSP2 by capillary endothelium inhibits the collagen degradation required for remodeling and causes dense fibers to form during development or wound healing by inhibiting matrix metalloproteinase (gelatinase) secretion (Sect. 8.3.3). Many synthetic implants directly promote TSP2

secretion and the formation of thick collagen fibers which have an avascular, poorly permeable capsule (Foreign Body Reaction). Delivering RNA complimentary to the processed mRNA.

In mice, TSP-3 slows (normalizes) the rate of vascular invasion and post-natal ossification of the cartilage during long bone development (Sect. 9.2.2) without affecting prenatal skeletal patterning. TSP-4 is an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions like fibronectin. TSP-5 contributes to the structures of cartilage and tendons by zinc or nickel ion-mediated binding to collagen.

Integrins determine the attachment of collagen fibers directly or through fibronectin, a multi-domain protein that also attaches collagen. Collagen binds to integrin by a different amino acid motif from that attaching to fibronectin. Direct collagen binding to integrin causes changes in fibroblast metabolism. Thrombospondins (TSP) are five independently encoded proteins unique to vertebrates. The family-1 thrombospondin-1 and -2 function differently. Thrombospondin-2 is expressed throughout the stroma where it mediates the development of a thick collagen fibrous network that walls off a foreign body such as a dental implant (foreign body reaction). Thrombospondin-1 activates transforming growth factor- β which stimulates fibroblast and osteoblast proliferation during connective tissue or bone remodeling and maintains the proliferation of dentally attached epithelial cells. The family-2 thrombospondins-3, and -5 promote cartilage development and its ossification to bone. The family-2 thrombospondin-4 mediates cell and stromal protein adhesion.

3.3.1. Stromal Nutrition

The fibroblasts and other cells of the stroma are surrounded by a dense layer of secreted materials through which nutrients must reach the cells and waste must be excreted. The arteriolar ends of blood capillaries have tiny junctions between the endothelial cells so that small molecules leak out under hydrostatic pressure. This fluid, *interstitial fluid*, feeds the stroma and then drains back into the venous end of capillaries under the influence of increased capillary osmotic pressure and reduced hydrostatic pressure. It contains glucose, amino acids, some metabolites such as citrate, pyrophosphate, and extracellular ATP (Sect. 9.1.4) as well as vitamins and inorganic ions. It is free of the proteins and other large molecules present in blood plasma, but it receives soluble proteins that are secreted into it by matrix cells such as fibroblasts.

The basal cells of a layered epithelium such as skin or gingiva (Sect. 5.2.1), or the periosteal cells covering bone, have such high nutrient demands that they need to be close to a rich capillary bed. Thus, although capillaries permeate the stroma, they are especially dense beneath the basal lamina of a layered epithelium (Sect. 13.3.1) or within a periosteum (Sect. 9.2.1). The gingival-tooth interface is especially susceptible to bacterial agents that

affect the subepithelial capillary plexus. The capillaries become leaky and exude traces of blood plasma and leukocytes into the gingival sulcus as an inflammatory exudate which cannot clot because of plasmin activation (Sect. 13.1.2 and Fig. 13.5a).

3.3.2.

Stromal Turnover, Inflammation, and Bone Loss

Hormones, infections and physical stresses promote degradation of the stroma by inducing affected cells to release *cytokines*. This *set of small proteins mediates cell-to-cell interactions, proliferation, differentiation, the activation of cell-specific organelles and diverse molecular pathways*. Some cytokines, called *interleukins*, are proinflammatory; they *increase capillary permeability* so that white blood cells pass into the stroma where their *lysosomes* are activated to secrete tissue destructive enzymes (Sect. 13.2.2). *Lysosomes* are membrane bound vesicles containing various acid-activated digestive enzymes. They are present in all cells, but are only activated if the cell has been damaged by physical or chemical injury directly, or by an infecting organism. The cytokines cause leukocytes to be extruded from nearby capillaries and activated to enhance lysosomal enzyme content at the site of damage or infection (Sect. 13.3.1). As the affected tissues are digested and removed, the surrounding and invading cells secrete *anti-inflammatory interleukins* and other substances that *promote acquired immunity and tissue repair*. A physiological example of cytokine action (Sect. 10.2.1) is the response to stress-induced micro-cracks that continually develop on bone surfaces.

Acquired immunity, for example antibodies that react to an antigen on the surface of an infecting bacterium, activate proteins called complement in the blood plasma. Complement proteins attract white blood cells (leukocytes) and activate them to digest (phagocytose) antigen-antibody complexes (Sect. 13.3.1). Complement proteins were named because they were initially determined to complement or complete the action of antigen-antibody complexes in the body. The interactions of antigen, antibody, and complement protect the body from specific bacterial infection and tissue damage.

A stromal matrix is permeated by interstitial fluid from capillaries. This fluid drains back into the capillaries except where the tissue is damaged, in which case the capillaries become leaky and proteins enter with the fluid, causing edema. Infections and physical stresses promote stromal degradation by inducing the cells to release proinflammatory cytokines that attract and activate blood granulocytes and macrophages. These cells secrete lysosomal, acid-activated, tissue destructive enzymes. As the infection or tissue damage is removed, the surrounding cells invade and start secreting anti-inflammatory cytokines that stimulate immunity and tissue regeneration.

This chapter describes the isolation and amino acid composition of the common type of collagen fiber polypeptides and how they associate into collagen fibers (Sect. 1). A description of how collagen fibers are stabilized by crosslinking then follows (Sect. 2). The non-fibrillar class of collagen is described, along with its role in modifying fibers and in forming filaments (Sect. 3). The chapter concludes with a detailed discussion of integrin proteins, with special emphasis on how integrins control fibroblast cell attachment to collagen fibers and collagen synthesis (Sect. 4).

4.1.1. Fibrillar Collagens

Collagen fibers are readily obtained from vertebrate bones, teeth, cartilage, ligaments, and dermis of the skin. Periodontal fibers from washed, extracted teeth may be cut out and the collagen fibers examined under a dissecting microscope. Collagen fibers in bones or dentin may be freed from the mineral by boiling them in disodium ethylene diamine until soft. Purified collagen fibers possess a unique amino acid composition: a third of the amino acids are glycine and another 23% are proline, of which almost half are hydroxyproline. Lysine comprises another 2 – 4% of residues, of which many are hydroxylysine. Short-chain amino acids such as alanine and serine total another 13% of residues (Table 4.1). Of the 20 encoded amino acids, tryptophan and cysteine are absent and the remaining 13 are present in small amounts. Because tryptophan is nutritionally essential, a diet consisting only of collagen cannot support life in humans or other mammals; nutritionally, collagen is said to have a biological value of zero.

Collagen fibrils, fibers and fiber bundles may be isolated from calf skin dermis by disaggregation with a neutral salt buffer followed by differential centrifugation. The pure fibers are boiled in sodium dodecyl sulfate (SDS) and subjected to polyacrylamide gel electrophoresis (SDS-PAGE). In this modern system, the collagen polypeptides are separated by molecular weight. Figure 4.1 shows that collagen fibers are composed of α_1 -, α_2 -, β -, and γ - polypeptides called tropocollagen. Marker proteins of known molecular weight indicate that the smallest polypeptides α_1 - and α_2 -tropocollagens are both approximately

Table 4.1 Amino acid composition of alpha-1 chain of tropocollagen

Important amino acid	% of total amino acids	Function
Glycine	33	Enhances van der Waals forces and hydrogen bonds that hold three helical polypeptides together.
Proline	14	Responsible for the extended helix.
Hydroxyproline	9	Hydroxyl group stabilizes the extended helix at higher temperatures.
Alanine	12	Small side chain allows polypeptides to lie alongside each other and form fibers.
Serine	4	Small side chain (same effect as alanine).
Lysine	3	Responsible for covalent cross-linking.
Hydroxylysine	1	Attaches carbohydrate and is involved with lysine in covalent cross-linking.

Percentage of total amino acids from Table 38.1, p 1134 of Principles of Biochemistry, White, A. et al., 6th Ed. 1978. McGraw Hill, New York

100 kDa; the α_1 -polypeptide being slightly larger because it migrates less. The origins of the β - and γ - chains are discussed below (Sect. 4.2.2).

The α_1 and α_2 -polypeptides may be cut out of the gel, each partially digested with a suitable protease and the fragments separated, blotted onto a membrane and the first 18–23 N-terminal residues of each peptide sequenced. After sorting for overlaps, large segments of the sequence may be obtained. The results consistently indicate that glycine is present at every third residue (Gly-X-Y repeating motif) in both α -polypeptides and that proline is often present at the X or Y position. Other studies indicate that artificial polypeptides containing a Gly-X-Pro repeating sequence have an extended (unfolded) chain due to the conformation of the proline peptide bond (Fig. 4.2), a left-handed extended helix called the *collagen helix* (Fig. 4.3) because of its presence in all types of collagen.

This left-handed secondary structure of collagen polypeptides differs from the α -helix, whose tightly coiled, right-handed helix is disrupted by proline. It also differs from a β -sheet, a series of six to ten amino acids in extended chain configuration in which proline residues create sharp turns, allowing the chains to lie alongside each other. The secondary structure of a fibrous collagen α -polypeptide is an extended helical rod (Fig. 4.4a and b). The glycine residues permit strong associations between the peptides (quaternary structure) due to hydrogen bonding between the hydrogen atom residue of glycine and carbonyl and amide groups of nearby peptide bonds. Tertiary structure is absent. The result is an extended triple helix, *tropocollagen* (Fig. 4.4c), which forms the monomeric unit of all fibrous collagens. Figure 4.5 is a cut-through view of the chains in Fig. 4.4. It shows a central cavity, across which the glycine hydrogen atom (glycine side-chain) holds the three α -polypeptides together by hydrogen bonding between alternate pairs of the chains.

Recently, fluoroproline, made by artificially replacing the hydroxyproline hydroxyl group with a fluoride atom, was found to form a collagen helix that was more stable to heat denaturation than hydroxyproline or proline. These studies also indicated that fluoroproline and hydroxyproline stabilize the collagen helix to heating by promoting an extended

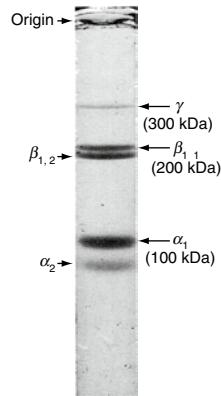


Fig. 4.1 The polypeptide composition of collagen fibers. Periodontal membrane fibers were dissected, boiled with sodium dodecyl sulfate (SDS), in the presence of mercaptoethanol to provide a reducing environment, and separated by polyacrylamide gel electrophoresis (PAGE). Polypeptides were visualized by staining with a dye after electrophoresis. The smallest polypeptides migrate most from the origin. The α -tropocollagen chains are about 100 kDa mol wt, the α_2 -chain being slightly smaller (nearer the foot of the gel) than the α_1 -chain. The β -bands have a molecular weight of about 200 kDa. The $\beta_{1,2}$ -band consists of an α_1 -chain covalently cross-linked to an α_2 -chain; the $\beta_{1,1}$ -band consists of two cross-linked α_1 -chains. The γ -tropocollagen chain has a molecular weight of 300,000 and consists of three covalently cross-linked α chains (Adapted from Fig. 2.7 published in: Biochemistry, A Case-Oriented Approach, 4th Edition. R. Montgomery, R.L. Dryer, R.C. Conway, and A. Spector, C.V. Mosby Co., St Louis, MO 1983; Copyright Elsevier, 2008)

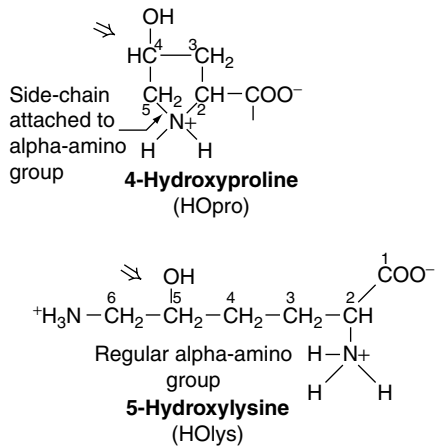


Fig. 4.2 Structures of hydroxyproline and hydroxylysine

(trans) form of the peptide bond. Other studies indicate that a proline helix becomes unstable above 10°C, but that replacing about half of the proline residues with hydroxyproline as in mammals (Table 4.1) stabilizes the helix at 37°C. In cold-blooded vertebrates such as fish, the tropocollagen must dissociate at 20°C or lower, not at 37°C. Less hydroxyproline is present in cold-water fish collagens. These studies suggest that it is the secondary

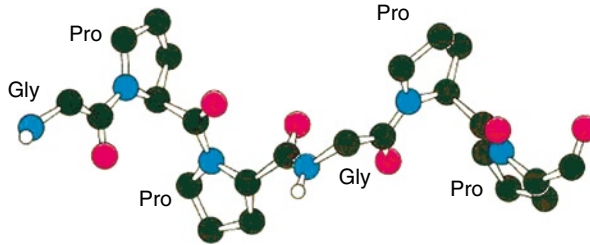


Fig. 4.3 Collagen helix. This secondary structure is created by peptide bond conformation around the proline and hydroxyproline residues (From Fig. 2-39 in Biochemistry. L. Stryer, 4th Ed. 1995. W.H. Freeman & Co., New York)

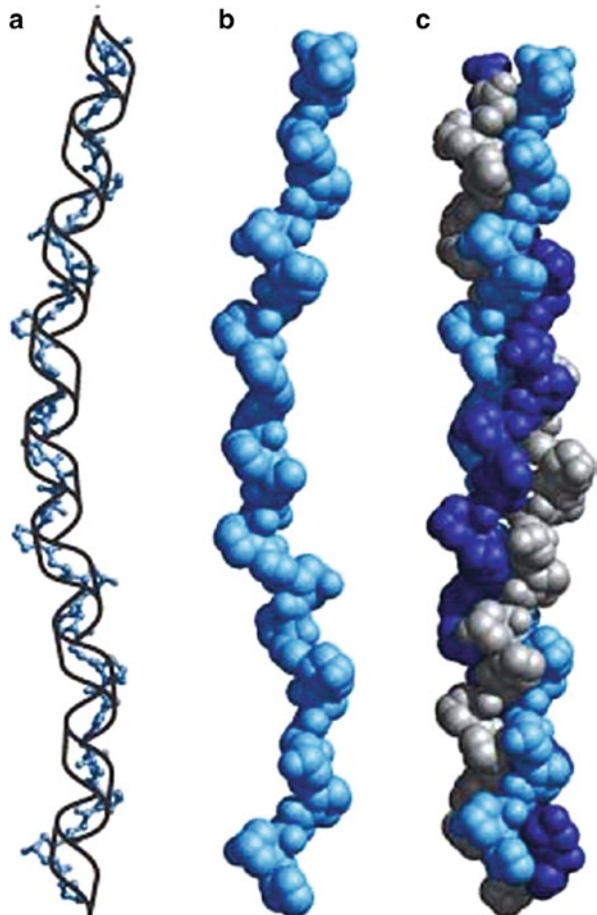
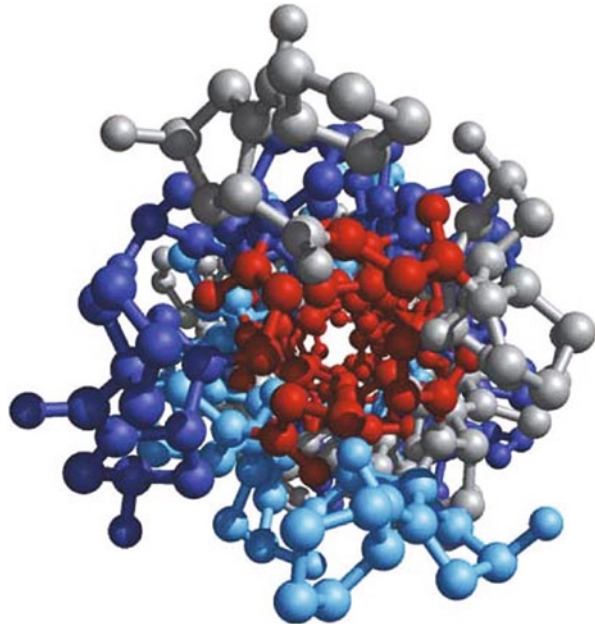


Fig. 4.4 Collagen triple helix formation.

(a) A repeating tripeptide sequence Gly-X-Pro or Gly-X-4-Hyp adopts a left-handed helical structure with three residues per turn. The repeating sequence used to generate this model is Gly-Pro-4-Hyp.

(b) Space-filling model of the same chain. (c) Three of these chains (*gray, blue, and purple*) wrap around one another with a right-handed twist (Figure 4.12a, b and c in Lehninger Principles of Biochemistry. D.L. Nelson and M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., New York)

Fig. 4.5 Glycine residues make up the interior of a tropocollagen triple helix. The same three-stranded collagen super-helix is depicted as in Fig. 4.4, but looking down the center of a ball-and-stick representation. Glycine residues ($-H$) are shown in red. Because of its small size, glycine is required where the three chains contact. The balls in this illustration do not represent the van der Waals radii of the individual atoms (Figure 4-12d in Lehninger Principles of Biochemistry. D.L. Nelson and M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., New York)



structure (the collagen helix) that stabilizes the triple helix to heat denaturation. Quaternary structure hydrogen bonds involving the OH group of hydroxyproline or the side chain of glycine to carbonyl groups of the peptide bond seem less important.

After its expression from fibroblasts, the α -polypeptides of tropocollagen form fibers spontaneously (without an enzyme). The standard model of tropocollagen is derived from peptides such as (pro-pro-gly) $_n$ that suggest a helical repeat length of 30 amino acid residues. Unfortunately, this finding is inconsistent with X-ray diffraction patterns from native collagens, which suggest a repeat length of only 21 amino acids. Indeed, recent analysis of nonpolar residues (Val, Leu, Ile, Met, and Phe) in the α_1 tropocollagen sequence of the most common form of collagen fiber reveals them to predominate near the centers of 21-residue segments. The spontaneity of collagen fibril assembly may be initiated by hydrophobic interactions between tropocollagen molecules at these centers within the extended chains and that this guides the subsequent, stabilizing formation of the glycine-bonded triple helix.

The alpha chains of fibrous collagens (tropocollagen) have many proline (and hydroxyproline) residues responsible for the collagen helix, an extended chain, left-handed helix (secondary structure) different from the α -helix and β -sheet in other proteins. Glycine and hydroxyproline are responsible for the association of the polypeptides into a triple helix. The glycine side chains (hydrogen atoms) hydrogen bond to carbonyl groups of a nearby peptide bond in the helical backbone and hydroxyproline OH groups

to amide groups of other nearby peptide bonds. The hydroxyproline bonds enhance triple helix formation at 37°C. Cold-blooded vertebrates have less hydroxyproline in their collagens (non-fibrillar as well as fibrillar). Serine and alanine have short side chains that allow the three chains to come together more easily than long residues. Recent X-ray diffraction studies of native collagen suggest that a 21 amino acid repeating unit whose central region contains hydrophobic amino acid residues initiates triple helix formation.

4.2.1. Collagen Fiber Formation

The α_1 - and α_2 - tropocollagen polypeptides are each a long, central portion (*domain*) of two larger polypeptides (*procollagen*) encoded by genomic DNA (genes *COL1A1* and *COL1A2*). The sequences of the respective genes are similar (homologous). And assemble so that two α_1 - and one α_2 -tropocollagen polypeptides interact by their hydrophobic domains and initiate triple helix formation. The tropocollagen polypeptides are then cleaved out as the fibril-forming monomeric unit (Fig. 4.6). The removed portions are called the *collagen propeptides*. The N- and C-terminal ends of the excised central domains are called the *tropocollagen telopeptide domains*. These telopeptide domains interact with adjacent tropocollagen molecules (yellow lines in Fig. 3.3) so that a stag-

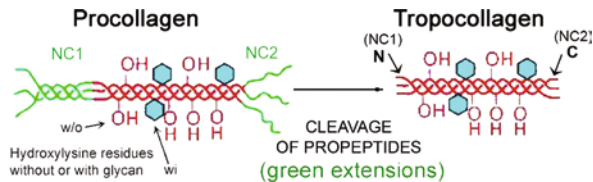


Fig. 4.6 Procollagen is the precursor of tropocollagen. Tertiary structures are absent from tropocollagen, but present on procollagen whose N- and C-termini fold like a regular protein. These globular non-collagenous (NC) domains at each end of the procollagen molecule (*green*) form soluble, tertiary structures that keep the central, glycine/proline-rich (collagenous) domain in solution until secreted from the cell. These domains are called *propeptides*. The C-terminal NC domain is usually referred to as NC1 and the N-terminal domain as NC2. In non-fibrillar collagens, and in fibrillar collagens with interrupted collagenous domains (see Table 3.1), there are additional NC domains which are numbered so that the C-terminus propeptides remains NC1. For example, the N-terminal domain of type IX collagen in cartilage is NC4 (see Fig. 6.14a). The propeptides are removed from fibrillar procollagens after secretion to form α -tropocollagen (*red, right side* of figure), but remain uncleaved in non-fibrillar collagens. The N- and C-terminal ends of α -tropocollagen are also non-helical and referred to as the *telopeptides* (N and C ends of *red portion* on *right side* of figure). Telopeptides are involved in cross-linking the fibers (see Fig. 4.9) (Modified from Fig. 19-47 in *The Molecular Biology of the Cell*, B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York)

gered array forms with gaps (bottom half of Fig. 3.3). The one-quarter overlap of the arrayed molecules causes a striated appearance, the dark and light banding pattern at the top of Fig. 3.3. The gaps also control how the fibers stain with dyes, cross-link, calcify and degrade (Table 4.2). Figure 4.7 shows how the quarter-staggered arrays aggregate into fibrils (thin, small fibers <30 nm diameter) and fibers (>300 nm diameter). Filaments and shorter length microfibrils are thin, extended aggregates (>10 nm diameter).

The extended triple-helical structure, repeated over and over in the quarter-staggered array, gives the collagen fiber its stiffness strength and insolubility. The helical wrapping of three polypeptides in tropocollagen provides greater strength than steel wire and the staggered array provides resistance to external forces. Heating to more than 90°C disrupts the hydrogen bonding between the polypeptides in a collagen fiber, and is analogous to the disruption of double-stranded DNA. The α -tropocollagen denatures and individual polypeptides are released from the fiber. The α -tropocollagen telopeptide domains cannot spontaneously reassemble into a triple helix because the excised propeptides are critical for helix formation. When cooled, the tropocollagen polypeptides lose their solubility and aggregate to form a clear gel. The denatured collagen (gelatin) is the basis of many manufactured food products and also an effective surface for in vitro cell and organ culture.

Table 4.2 Functions of gaps in the fibrillar collagen array

1.	Dye staining gives the microfibril a striated appearance under the electron microscope.
2.	Lysine residues predominate around the gaps, causing glycan attachment and polypeptide cross-linking.
3.	Phosphorylation nucleates calcium as bone forms.
4.	Site of initial collagenase degradation.
5.	Site of fiber interactions with non-fibrous collagens

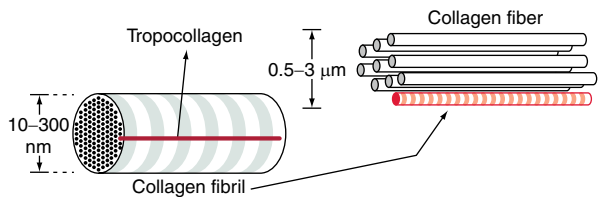


Fig. 4.7 Super-arrays of tropocollagen form collagen fibrils or fibers. Each fibril (*left*) is composed of a 10×300 nm bundle of α -tropocollagen monomeric units that have aggregated in quarter-staggered array. A single tropocollagen polypeptide is indicated as a red arrow and the ends of the others in the fibril are seen as black dots (*far left*). Collagen fibers in the periodontium or a tendon are made up from bundles of fibrils that are 50 times thicker and 300 times longer (*right*) (Adapted from Fig.19-47 in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York)

4.2.2.

Fiber Cross-Linking: Formation of β - and γ -Tropocollagen

The tropocollagen β - and γ -chains in Fig. 4.1 are respectively twice and three-times the size of the α -chain. Indeed, their amino acid composition is virtually identical to the α -chain in Fig. 4.1, except for the appearance of derivatives of lysine and hydroxylysine, mainly lysyl hydroxyl-norleucine and pyridinoline. They increase in amount with age of the organism.

Along with procollagen, fibroblasts secrete an enzyme, lysyl oxidase, which binds to and activates oxygen gas with copper ions at its catalytic site. Lysyl oxidase attaches to gaps in the fibrils where it converts side-chain terminal amino group of lysine or hydroxylysine residues (ϵ -amino group) within the tropocollagen telopeptide domains into an aldehyde, *allysine*, (α -aminoadipic- δ -semialdehyde) or *hydroxyl-allysine* (hydroxy- α -aminoadipic- δ -semialdehyde). Subsequent cross-linking reactions are all spontaneous and due to the reactivity of the generated aldehyde group. The allysine or the hydroxyallysine aldehyde group reacts with the ϵ -amino group of lysine or hydroxylysine in an adjacent polypeptide to cross-link two polypeptides, forming dehydro-hydroxylysinorleucine or dehydro-lysinorleucine cross-links (Fig. 4.8). The illustrated reaction between allysine and hydroxylysine is more frequent than between allysine and lysine, but either gives rise to β -tropocollagen in which the lysine-derived cross-link is stable. Some of the dehydro-hydroxylysinorleucine or dehydro-lysinorleucine molecules are reduced in vivo to hydroxylysinorleucine or lysinorleucine.

γ -Tropocollagen forms when an α -polypeptide becomes linked to two others by separate dehydro-hydroxylysinorleucine bonds, or by dehydro-hydroxylysinorleucine being transformed into pyridinoline through adding a third allysine residue from another α -chain (Fig. 4.9). Pyridinoline cross-links appear mostly in the telopeptide region of tropocollagen (Sect. 4.2.1), but few are present and the γ -chain band in Fig 4.1 is faint. The older the organism, the greater is the extent of collagen cross-linking. Collagen fibers from young animals contain few β - and almost no γ -polypeptides, whereas the fibers from old animals have substantial amounts of β -polypeptides and some clearly detectable γ -polypeptides.

Cross-linking contributes to tissue strength and limits the need for fiber replacement, but it also inhibits repair following a mechanical injury or infection (Sect. 8.1.3.). Lysyl oxidase catalysis is self-limiting to avoid excessive cross-linking. The oxidation rate of lysine amine residues is limited to approximately 100 catalytic turnovers per enzyme molecule because ammonia and other reaction by-products inactivate it irreversibly.

Collagen fibers must be stable for some years for measurable amounts of pyridinoline to form. Because bone is the major source of long-term stable collagen fibers, *an increase in peptides containing pyridinoline in the blood or urine is a good marker of increased bone remodeling and resorption*. Normally, amounts of these peptides are low except at adolescence and in women shortly after menopause (Fig. 4.9). Large fluctuations in collagen turnover outside of bone make measurements of hydroxyproline in the blood or urine an unreliable measure of the extent of collagen degradation. Most hydroxyproline is metabolized in the

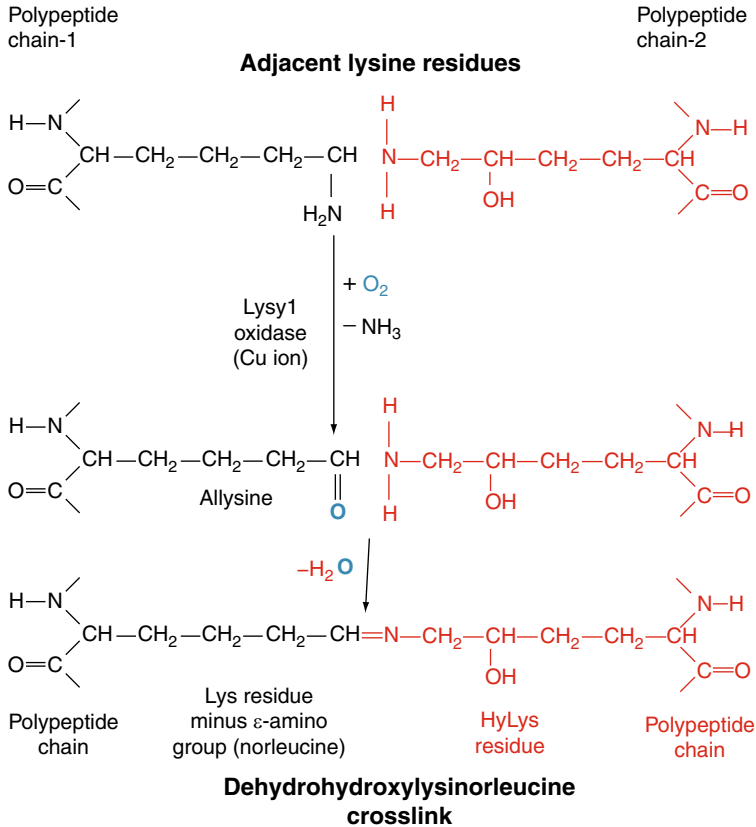


Fig. 4.8 Formation of β -polypeptides from tropocollagen. The ϵ -amino group of a lysine residue within a tropocollagen polypeptide (chain 1, colored black) is oxidized to an aldehyde by lysyl oxidase, an enzyme secreted by fibroblasts. This enzyme adheres to the gap regions of the quarter-staggered arrays and uses molecular oxygen (blue) which reacts with cupric ions at the catalytic center of the oxidase. One oxygen atom oxidizes the ϵ -amino group of an adjacent lysine residue, spontaneously driving out ammonia (NH_3). The aldehyde reacts spontaneously with a nearby hydroxylysine or lysine residue on another chain (chain 2, red) to form a dehydro-hydroxylysinorleucine (or dehydro-lysinorleucine) crosslink. Because, the peptide is reduced on hydrolysis with 6.0 M HCl to detect these cross-links, the products found are always hydroxylysinorleucine or lysinorleucine (not the dehydro forms). It is likely that some cross-links remain in the dehydro- form in vivo and that others are reduced (Adapted from the diagram at the foot of p.128 in Lehninger Principles of Biochemistry. D.L. Nelson and M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., New York)

liver to 4-hydroxy 2-ketoglutarate and ultimately to glycollate and glycine (or glyoxylate). By contrast, dehydro-hydroxylysinorleucine and dehydro-lysinorleucine are reduced to hydroxylysinorleucine and lysinorleucine and returned to blood plasma where they are reliable measures of pathology (See also, Sect. 10.1.3).

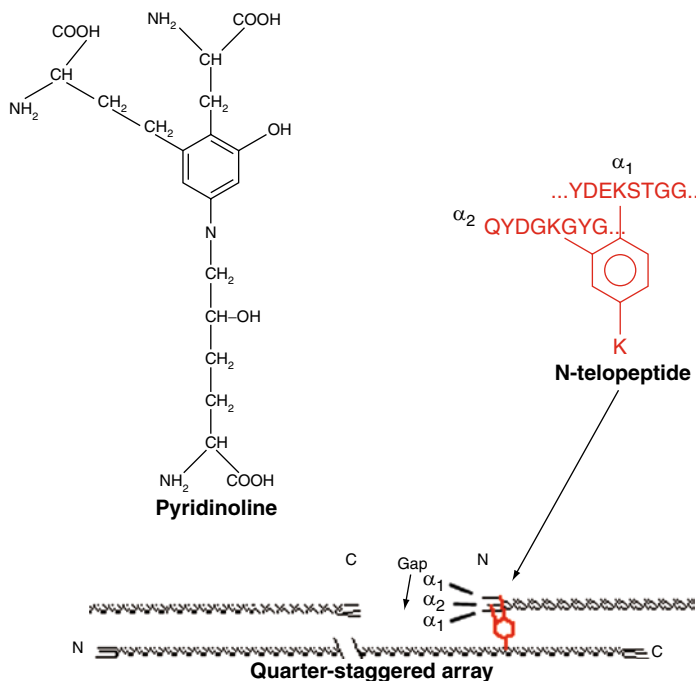


Fig. 4.9 Formation of pyridinoline from tropocollagen. Dehydro-hydroxylysinorleucine forms at the N- (or C) telopeptide region near a gap region. Pyridinoline (*top left*) is formed when a lysine residue from a third polypeptide at this site is oxidized by lysine oxidase and becomes covalently bonded to the dehydro-hydroxylysinorleucine. The orientation of the three polypeptides that form pyridinoline is shown in the quarter-staggered array at the foot of the figure. When collagen fibers are degraded *in vivo*, two telopeptide sequences are incompletely degraded (single letter sequences), but the third polypeptide is removed except for its attached lysine residue. (*top, right*) (Slightly modified from Figs. 1 and 3, D.A. Hanson, "A Specific Immunoassay for Monitoring Human Bone Resorption: Quantitation of Type I Collagen Cross-Linked N-telopeptides in Urine." J. Bone Miner. Res. 7(11): 1251–1258, 1992. Also Fig. 3, Ureña, Pablo and Marie-Christine de Vernejoul. 'Circulating biochemical markers of bone remodeling in uremic patients.' Kidney International. 55:2141–2156 (1999))

Tropocollagen alpha chains are encoded as parts of larger polypeptides from which the N- and C-terminal regions (propeptides) are cleaved. The wrapping of three α -polypeptides into a triple helix, repeated over and over, gives the collagen fibers their stiffness, strength and insolubility. The triple helical monomeric tropocollagen molecule polymerizes into a quarter-staggered array, forming gaps that are critical for staining, cross-linking, calcification and degradation. Covalent cross-links between α -polypeptides strengthen the fiber and are caused by the co-secretion of lysyl oxidase, near gaps in the collagen array. Lysyl oxidase uses molecular oxygen and copper ions to oxidize the terminal ϵ -amino groups of a lysine residue near the

N- or C-terminal end of an α -polypeptide (telopeptide region). The lysyl aldehydes (allysines) spontaneously but slowly interact with nearby ϵ -amino lysine residues on the same or different polypeptides. The result is covalent cross-linking between two or three α -polypeptides (β - or γ -chains) that increases whose amounts in collagen fibers increase with age. The covalent links are dehydro-hydroxylysyl-norleucine (β - or γ -chains), or pyridinoline (γ -chains). Pyridinoline mostly forms in bone whose collagen is least remodeled. The blood plasma content of peptides containing pyridinoline and hydroxylysyl-norleucine are good indicators of collagen degradation.

4.3.1. The Collagen Superfamily

Although by far the most abundant, fibrillar collagens comprise only five of the 27 collagen types in the body, the remainder being non-fibrillar. All collagens are made from procollagen monomeric units composed of three polypeptides in a triple helix (Fig. 4.6). Proteins that contain a short tropocollagen-like sequence are not collagens. Each type of collagen is made up from identical or different polypeptides encoded in the genome. As noted also in Table 3.1, the different types of collagen polypeptides are numbered in Latin characters (I, II, etc.) and correspond to different gene products. A triple helix may be composed of a single polypeptide as in type II procollagen [$\alpha(\text{II})_1$]₃ and encoded as a single gene (COL2A1). Alternatively, a triple helix may be composed of two polypeptides as in type I procollagen [$\alpha(\text{I})_1$]₂, $\alpha(\text{I})_2$, which is encoded as 2 genes (COL1A1 + COL1A2), or of three different polypeptides as in some type IV procollagens, $\alpha(\text{IV})_3$, $\alpha(\text{IV})_4$, $\alpha(\text{IV})_5$ encoded as 3 genes (COL4A3 + COL4A4 + COL4A5). The different polypeptide sequences of the different collagen types therefore give rise to different structures.

4.3.2. Fiber-Modifying Non-fibrillar Collagens

Only five procollagens: types 1, II, III, V and XI, are processed to tropocollagen. Type II fibers are unique to cartilage and are limited in thickness by complexing with types XI and type IX collagens (Table 3.1). Other collagen types are expressed in small amounts and they influence the fiber thickness and shape of type I collagen, or anchor a group of fibers to each other and the surrounding tissues.

Type III fibers (fetal, reticular and vascular collagen) are delicate compared with type I fibers. In the fetus, type III collagen is incorporated within the type I collagen to impart the greater flexibility critical for fetal development. After birth, the delicate type III collagen fibers contribute to reticular fibers and also type I collagen fibers that are present in cardiovascular and lymphoid tissues and also beneath epithelial basal cell layers, muscles, and nervous tissue Schwann cells.

In the cornea and lens of the eye, the presence of small amounts of type V collagen around type I fibrils adjusts their orientation so that they are translucent. In developing long bones, a network collagen, type X, is induced to attract blood vessels that invade cartilage prior to its transformation into bone (Sect. 9.2.2.). The blood vessels bring osteoblasts, which replace the resorbed type II collagen of the cartilage with type I collagen. In children and adolescents, the site of this replacement and growth, the *epiphyseal growth plate*, is almost exclusively composed of type X collagen.

4.3.3.

General Structure of Non-fibrous Collagens

Non-fibrillar collagens are composed of polypeptides that have sequences of glycine, proline and hydroxyproline residues as in fibrous collagens and also form a triple helical monomeric unit. However, the helical region is shorter or interrupted and the C- and N-terminal propeptides, except for the short N-terminal secretory signal sequence (pre-propeptide sequence), are not removed. As in fibrous collagens, the propeptides possess a non-collagenous (NC) structure and, in addition to controlling triple helix formation, they control how the triple helical units (protomers) aggregate. The non-fibrillar collagens form basal laminas (i.e. basement membranes), anchors, and microfibrils (Table 3.1). The N and C-terminal regions of the triple helices are not cleaved off like fibrous collagens, nor are they cross-linked by oxidized lysine residues. Indeed, the major cross-links found in types IV, VII and XVII collagens are mediated by cysteine-disulfide bonding, mostly within or close to the C-terminal propeptide regions. Some relevant non-fibrous collagen structures are discussed in Chapter 5.

4.3.4.

Beaded Collagen Filaments

Microfibrillar collagen (type VI) and elastic microfibrils called oxytalan fibers (described in Sect. 3.1.3) are beaded filaments (sometimes called microfibers). These two types of beaded filaments in the connective tissue stroma are *collagen type VI*, and *fibrillin* with or without *elastin*. Collagen type VI filaments are non-stretchable, whereas fibrillin filaments are stretchable. Type VI collagen forms small diameter beaded microfilaments that interweave among large collagen fibers by crossing the gap regions of the type I and type III fibers (Table 4.2). These filaments strengthen collagen fiber resistance to mechanical forces beneath the dermis and also around arteries and capillaries (Sects. 3.1.3 and 11.1.1). The collagen filaments are composed of three separately encoded α -polypeptides with similar length triple helical domains but different sizes of N- and C-terminal non-helical domains (illustrated at the top of Fig. 4.10). It is commonly observed as a microfibril with a double-beaded period of about 100 nm due to aggregated non-collagenous C- and N-terminal domains. The formation of a typical collagenous beaded filament is illustrated in Fig. 4.10 and its legend.

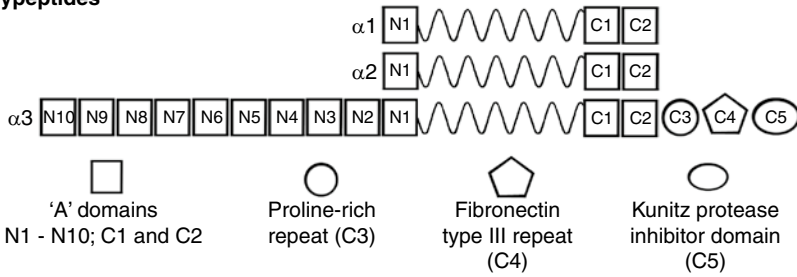
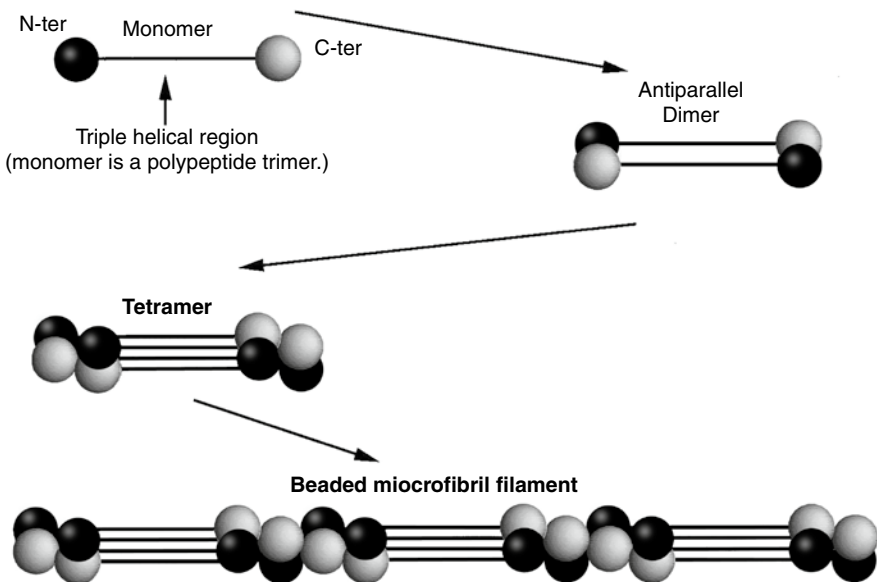
Polypeptides**Assembly**

Fig. 4.10 Beaded filaments of type VI collagen. *Top*: Diagram of the 3 α -polypeptides with their central collagenous domain (wavy line) connecting non-collagenous N- and C-terminal regions squares indicating the various domains in the N- and C- terminal non-collagenous domain. The non-collagenous N- and C-terminal domains (C1 and C2) are Von Willebrand Factor (VWF) “A” domains (Fig. 11.2). Domains C3, C4, and C5 are unrelated domains described in the figure. *Bottom*: Assembly from a monomeric protomer, a collagen triple helix with a massive non-collagenous domain at each end (bar-bell model). The helical region is interrupted, allowing the triple helical domain to twist into a segmented super-coil (not shown). The N- and C-terminal ends adhere to each other to form an anti-parallel dimer. Two dimers adhere similarly to form a tetramer that extends laterally such that only N-terminal ends adhere to C-terminal ends (*bottom left*). The massive non-collagenous domains form the beaded filaments and are stabilized by intra- and intermolecular disulfide bonds (From Fig. 2 in Knupp C and Squire JM (2005) “Molecular Packing in Network-Forming Collagens.” *Adv. Prot. Chem.* 70:375–403. Assembly is from Fig. 3 in Fauvel-Lafève F (1999) “Microfibrils from the arterial subendothelium.” *Int. Rev. Cytol.* 188:1–40). Figure was modified by Dr Wirsig-Weichmann

The collagen superfamily has two major classes, fibrillar and non-fibrillar, based on their gene sequences and how the polypeptides are processed and assembled. There are more than 27 collagen genes, of which only five are fibrillar. The fibrillar collagens, especially type I are the most common. Although each monomeric unit is composed of a specific tropocollagen type, a mixture of different types influences assembly in a given tissue. The non-helical N- and C-terminal regions are removed from all fibrillar collagens, but remain intact or mostly intact in all non-fibrillar collagens. Microfibrillar collagen (type VI) and elastic microfibrils called oxytalan fibers are beaded filaments or microfibers. Beaded collagen fibers composed of type VI collagen strengthen larger type I and type III collagen fibers in the dermis and around arteries and capillaries by binding at the gap regions of the quarter-staggered array of the latter collagens. The large non-collagenous N- and C-terminal regions of type VI collagen interact with the triple helices and aggregate into antiparallel tetramers. The tetramers form beaded filaments by end-to-end aggregation.

4.4.1. Integrins and Regulation of Stromal Composition

Integrins are a family of transmembrane proteins that control when a cell expresses connective tissue matrix components, or responds to environmental stresses. Integrins are heterodimeric receptors composed of one α - and one β -polypeptide. Each heterodimer is a single molecule with two sites: an extracellular receptor site and an intracellular signaling site. The former is the *N-terminal head region* which binds to extracellular proteins such as collagen, fibronectin or laminin. The latter is the *C-terminal tail region* which remains on the cytosolic side of the membrane and interacts with cytoplasmic effectors. Integrin signaling is passed to the cell by extracellular ligands (*outside-in* signal), or from the cell by intracellular ligands (*inside-out* signal). Human integrins are selected from one of 18 α -polypeptides and one of 8 β -polypeptides, although only 24 of the 144 combinations exist (Fig. 4.11). The β -polypeptides (~90 kDa) are smaller than the α -polypeptides (~130 kDa).

Flexible domains influence whether an extracellular ligand will bind to an integrin. The domains are listed and illustrated in Fig. 4.12a. Integrins exist in three major conformations (Fig. 4.12b): i) low-affinity (inactive); ii) ligand-bound (activated) or iii) high-affinity (primed). The change from inactive to activated conformation is mediated by a large global rearrangement whereby the integrin subunits extend with a sharp, 'switchblade'-like motion. A ' β -propeller' domain (Fig. 12a) is N-terminal in about half of the α -polypeptides, but an additional domain extends from two blades of the propeller, the *inserted domain* (I-domain) in the remainder. The I-domain is homologous to the N-terminal domain of the β -polypeptide, which is called the *I-like domain*. Four of the 9 human α -polypeptides possessing an I-domain (α_1 , α_2 , α_{10} and α_{11}) bind collagens when partnered with a β_1 -polypeptide.

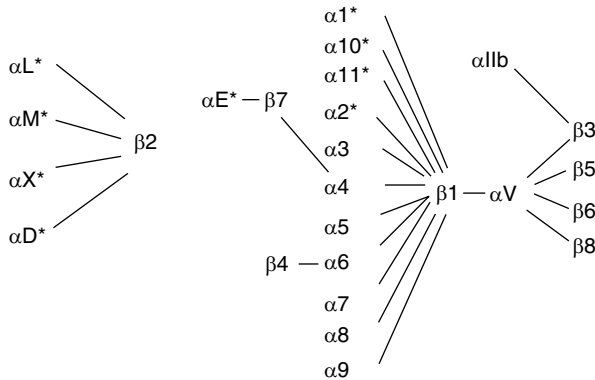


Fig. 4.11 Integrin subunit combinations. Integrin α - and β -subunits form 24 heterodimers that recognize distinct but overlapping ligands. Half of the α -subunits contain I-domains (*asterisks*). The β_2 integrin subunit is also called CD18 (Cell Differentiation antigen 18) and its various α -subunits are called CD11a, b, etc. The α - and β_2 -subunit and its partners are important in lymphocyte adhesion and activation, as discussed in Sect. 13. 2.4. (Reprinted from *Advances in Protein Chemistry*, Volume 68, Springer, TA and Wang, J-H., The three-dimensional structure of integrins and their ligands, and conformational regulation of cell adhesion, pp 29–63, 2004, with permission from Elsevier)

In integrins whose α -polypeptides do not possess an I-domain, the β -propeller domain contacts the I-like domain of the β -subunit when the conformation is extended (Fig. 4.13a). Ligands such as laminin (Sect. 5.1.1) are attracted to the extracellular integrin surface where the RGD aspartic acid residue in the consensus motif (Sect. 3.2.1), or a glutamic acid within other integrin-binding motifs, open a *metal ion dependent adhesion site* (MIDAS), exposing a divalent cation (usually Ca^{+2}) within the *I-like domain*. Exposing the cation moves the C-terminal helix of the I-like domain down (white arrow in Fig. 4.13a) which stabilizes the integrin-ligand complex by causing an outward swing of the hybrid domain of the β -polypeptide (curved black arrow in Fig. 4.13a).

In α -polypeptides possessing an I-domain, the mechanism is more complex because the I-domain first interacts with the I-like domain to stabilize a binding site that is not RGD-dependent. Contact with collagen (or another ligand) activates a glutamic acid residue in a consensus motif on the ligand to expose the I-domain's MIDAS site containing Mg^{2+} or Mn^{2+} . The C-terminal helix of the I-domain is pushed down (upper white arrow in Fig. 4.13b), exposing an acidic amino acid that binds to a Ca^{+2} ion in the MIDAS site of the I-like domain (lower white arrow in Fig. 4.13b). The β -polypeptide's hybrid domain then swings out as in integrins without an I-domain (curved black arrow).

Cytoplasmic effectors can bring the cytoplasmic ends closer, closing the metal binding site of the I- or I-like domain and releasing the ligand. If effectors bring the cytoplasmic domain still closer, they cause the integrin to collapse to the bent state (Fig. 4.12b). Conversely, other cytoplasmic effectors can promote integrin binding to extracellular ligands by causing the cytoplasmic ends to move apart.

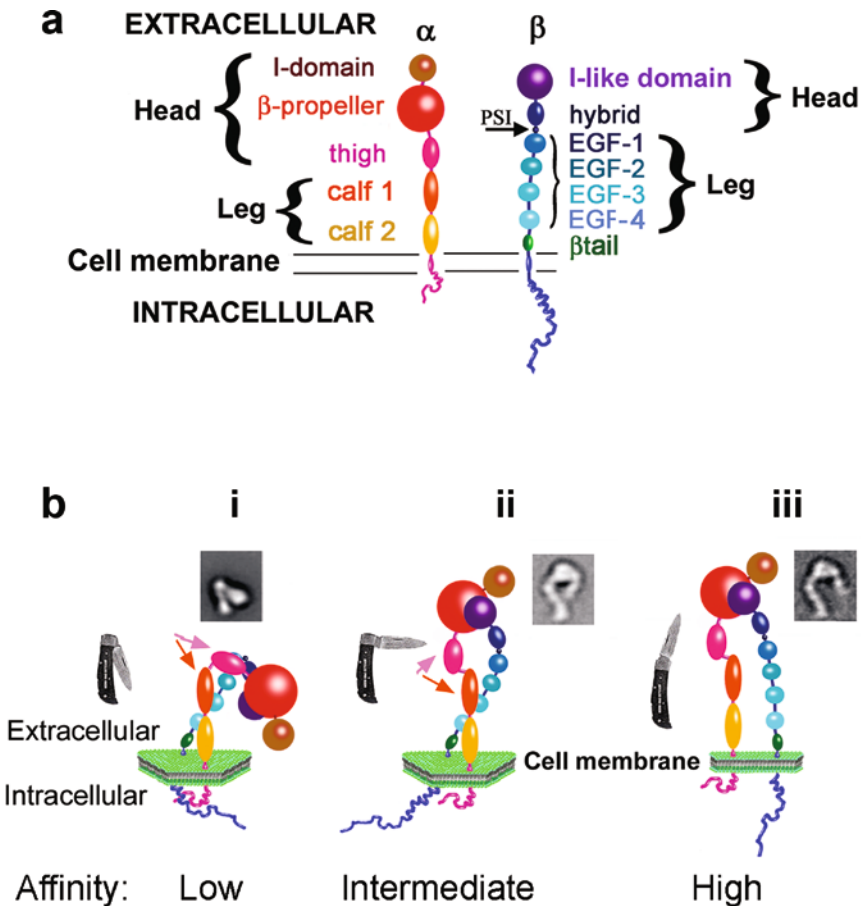


Fig. 4.12 Domain structure and integrin conformations. **(a)** The α - and β -polypeptide domains. In α -polypeptides, the N-terminus is a β -propeller domain (red) made up from seven β -sheets interwoven into a seven bladed propeller, or an *inserted domain* (I-domain; brown) that extends out from the N-terminal β -propeller (see text). Beneath the propeller are the thigh domain (maroon), and two leg domains, calf 1 (orange) and calf 2 (yellow). The region between the thigh and calf 1 domains is flexible and the thigh and propeller domains (with or without an I-domain) comprise the α -polypeptide head region. In β -polypeptides, the N-terminal domain is homologous to the I-domain (I-like domain; purple). The I-like domain is attached to a hybrid fold related to a fold in immunoglobulins (dark blue), followed by 4 cysteine rich EGF domains (shades of blue) similar to those in fibrillin (Sect. 6.1.1) but not calcium-ion binding. The EGF domains are followed by a β tail domain, and a single transmembrane helix that anchors each polypeptide within the lipid membrane bilayer. Between the hybrid and first EGF domain of the β -polypeptide is a flexible region, the plexin-semaphorin-integrin (PSI) region, named for common sequence homologies. The I-like and hybrid domains form the β -polypeptide head region. The respective domain structures are stabilized by intra-chain disulfide bonding. Both polypeptides possess a C-terminal intracellular region that interacts with cytoplasmic signaling components. **(b)** Conformations of the α (α) and β (β) subunits. Integrins possess bent, partially extended and fully extended conformations analogous to those of an almost-closed, partially open, and fully open switchblade

The *collagen binding site* for integrins is a short flexible region (Gly-X-X-Gly-Glu-Arg where X is any amino acid). This motif is conserved in all fibrous and some non-fibrous collagens, especially types IV and VI. Different amino acid sequences (at the N-terminus of each integrin α -polypeptide) determine which type of collagen will bind. For instance, the I-domain of α_2 - or α_{10} -polypeptides attaches fibrillar collagens, whereas the I-domain of α_1 - or α_{11} -polypeptides attaches non-fibrillar collagens. Each of these integrin α subunits have different cytoplasmic partners that result in different cell responses when the appropriate ligand binds. Fibroblasts secreting collagen are long and thin and do not divide whereas dividing fibroblasts are rounded up and release any integrin-bound stromal proteins. This difference is mediated by growth factors or by small cell-signaling proteins called cytokines that produce ligands that determine whether the cytosolic ends of each integrin polypeptide can associate. Collagen fibers are synthesized only when the cell cannot divide.

A few integrin α - and β -polypeptides bind multiple ligands. For instance the $\alpha\text{M}\beta_2$ integrin (Mac-1 receptor of neutrophils and macrophages (Sect. 13.2.4) has a ligand binding region (Lys245 – Arg261) that can attach more than 30 different proteins including laminin, collagen fibers and the fibrin of clotted blood (Sect. 11.3.1). Mice made deficient in integrin α -polypeptides that bind laminin and some other connective tissue components do not survive. However, mice deficient in α_1 or α_2 subunits appear normal and only exhibit a defective fibroblast response to injury. Table 4.3 lists the integrins that bind to collagen.

Table 4.3 Functions of collagen binding integrins

Integrin	Function
$\alpha_1\beta_1$	induces fibroblast proliferation and decreases collagen synthesis in response to cytokines in angiogenesis, fibrosis, chronic inflammation and bone healing
$\alpha_2\beta_1$	stromal protease activation (remodeling) in response to stress or injury with de novo synthesis of both collagenase and collagen
$\alpha_{10}\beta_1$	expressed on chondrocytes and influences cartilage maturation
$\alpha_{11}\beta_1$	skeletal development around vertebral cartilages and fibers

←
knife. *i. Bent (low affinity).* The region between the calf 1 and thigh region of the α -polypeptide (arrows) bends along with the PSI region of the β -polypeptide. Both polypeptide head regions lose ligand binding and face the calf 2 domain. The intracellular (C-terminal) regions of the α - and β -polypeptides overlap. *ii. Partially extended (closed head; intermediate affinity).* The two intracellular regions partially separate, the α -polypeptide calf 1 to thigh connection (arrows) straightens up and the β -propeller of the α -polypeptide (red) pulls up the I-like domain of the β -polypeptide (purple) straightening the PSI domain. *iii. Fully extended (open head; high affinity).* The PSI region of the β -polypeptide rotates around the around the α -polypeptide calf domains. The movement opens the binding site and also completely separates the two intracellular regions (Fig. 4.13). Fig is based on Fig. 1 in Carman CV, Springer TA (2003 Oct) “Integrin avidity regulation: are changes in affinity and conformation underemphasized?” Current Opinion in Cell Biology, 15(5): 547 – 556. (Modifications were by Dr Wirsig-Weichmann)

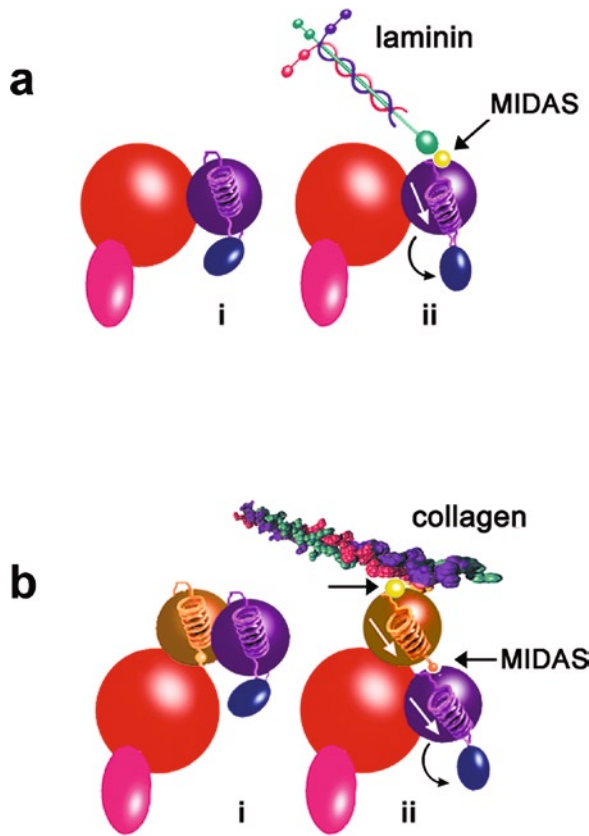


Fig. 4.13 Ligand-integrin receptor interactions. Only the head regions are shown. The presence or absence of an I-domain in the α -polypeptide affects the mode of receptor activation. **(a)** *I-like domain receptor*. (i) The propeller domain of the α -polypeptide (red) interacts with the I-like domain of the β polypeptide (purple) and the MIDAS cation making up the ligand receptor site (see text), but the site remains closed. (ii) An acidic amino acid in the ligand causes a downward displacement of the seventh and most C-terminal α -helix within the I-like domain (α -7 helix), opening the ligand binding site and stabilizing it with an outward movement of the hybrid domain of the β -polypeptide (curved black arrow). **(b)** *I-domain receptor*. (i) A MIDAS site cation in the I-domain interacts with the I-like domain to open and stabilize the ligand binding site. In these integrins, the I-domain MIDAS possesses Mg^{+2} or Mn^{+2} , and the I-like domain MIDAS possesses Ca^{+2} (see text). (ii) The ligand-binding, high-affinity conformation of the I-domain is opened by ligand binding (first downward-pointing white arrow), and stabilized by a downward displacement of the C-terminal α -7 helix containing an acidic residue that binds to the Ca^{+2} ion in the I-like domain (second downward-pointing white arrow). This movement stabilizes the I-like domain by attaching it to the propeller domain along with an outward movement of the hybrid domain of the β -polypeptide (curved arrow as in a). Fig is based on Fig. 1 in Carman CV, Springer TA (2003 Oct) "Integrin avidity regulation: are changes in affinity and conformation underemphasized?" *Current Opinion in Cell Biology*, 15(5): 547–556. (Modifications were by Dr Wirsig-Weichmann)

Despite all possessing the β_1 subunit and a limited range of α subunits, the effects on collagen synthesis and degradation vary greatly. Integrin α_{11} is also strongly expressed with β -subunits other than β_1 in mature tendon and ligament cells, but the signaling properties of this integrin combination are as yet uncertain.

Integrins are composed of transmembrane heterodimers with an N-terminal, extracellular binding site for a ligand such as laminin or collagen, and a C-terminal intracellular binding site for a cytosolic activating or deactivating proteins. The ligand binding site is activated by a “switch-blade” change in conformation, from bent to activated, to primed. Presentation of the binding site is determined by stromal cytokines and growth factors that influence cytosolic factors, or by intracellularly induced cytosolic factors. Binding to ligand: (1) strengthens cellular attachment to the extracellular matrix; (2) induces specific changes in fibroblast metabolism; and (3) affects growth factor and cytokine signaling involved in mesenchymal tissue development, maintenance, and repair. Changes in the cell environment mediate changes in the approximation of the cytosolic ends of the heterodimers that close or open the “switchblade.”

This chapter describes the organization of the major proteins that form a basal lamina which connects an epithelium to its underlying stroma (Sect. 1). The organization and major protein composition of oral and gingival epithelium and the junctional epithelial dental attachment at the base of a gingival sulcus are described (Sect. 2).

5.1.1.

Basal Lamina and its Stromal Attachment

An *epithelium* such as the skin or oral mucosa is separated from the underlying stroma by a basal lamina. The basal lamina is derived from both epithelium and connective tissue, but its major components are induced from connective tissue fibroblasts by the overlying epithelial cells. All basal laminas consist of two parts (Fig. 5.1): a lightly staining layer containing *laminin*, the lamina lucida which contacts basal epithelial cells; and an intensely staining layer beneath that contains *type IV collagen* (lamina densa) and contacts the collagens and other proteins of the stroma.

Laminins are composed of three polypeptides, α , β , and γ that are homologous and encoded by separate genes in the mammalian genome. What genes are expressed is determined by tissue type. There are five different types of α chain, three types of β chain, and three types of γ chain, but fewer laminins than integrins (Sect. 4.4.1). The major laminin in the lamina lucida is laminin-1 ($1\alpha, 1\beta, 1\gamma$; Fig. 5.2) which is secreted by fibroblasts, but its minor laminin, laminin-5 ($5\alpha 1\beta 1\gamma$) is secreted by epithelial cells. The nomenclature for laminins was recently changed to indicate which of the alpha, beta and gamma chains are used in a particular laminin. Thus, the two laminins referred to above are now written laminin-111 and laminin-511. The older laminin nomenclature is used in this book. Laminin-1 assembles into a clear, web-like polymer (Fig. 5.2) above the lamina densa, whereas laminin-5 forms long, thin strands that pass through the lamina lucida and bind to type IV collagen in the lamina densa. A few laminin-5 strands pass right through lamina lucida and lamina densa and become attached to the head region of *type VII collagen* anchoring fibrils (see end of Sect. 5.1.2).

Type VII collagen is a non-fibrous, anchoring fibril that binds large type I collagen fibers in the stroma to the lamina densa section of the basal lamina. Type VII procollagen

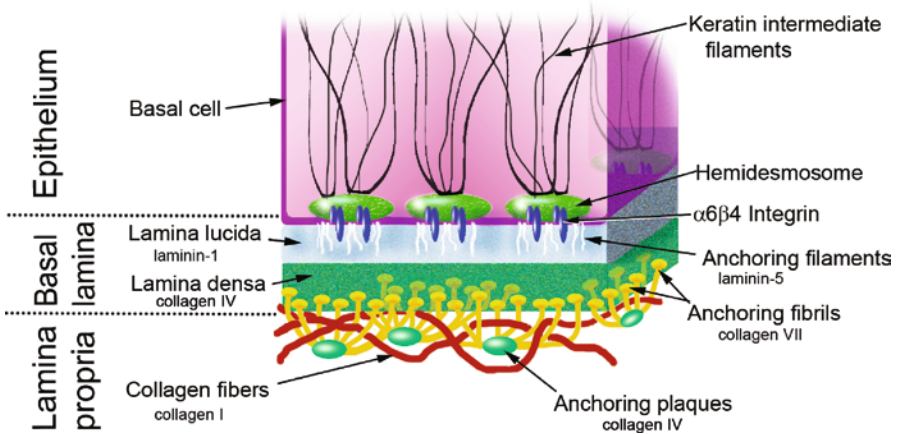


Fig. 5.1 Diagram of a basal lamina. The various components are shown with reference to histological markers (*bold*) and their major biochemical components (*small print*). The lamina lucida and lamina densa are the *light blue* and *dark green* regions beneath a basal cell (epithelial or epidermal cell). The lamina lucida is usually composed of a laminin-1 network (*light blue*) within filaments of laminin-5 (*white*) (From Fig. 4 in Giehl KA, Ferguson, DJP, Dean D, Chuang, YH, Allen J, De Berker, DAR, Tosti, A., Dawber RPR and F. Wojnarowska, F. Alterations in the basement membrane zone in pili annulati hair follicles as demonstrated by electron microscopy and immunohistochemistry. *British Journal of Dermatology*, 150:722–727, 2004. With copyright permission from Wiley-Blackwell, PO Box 805, 9600 Garsington Road, Oxford OX4 2DQ, UK, as modified by Dr Wirsig-Wiechmann.)

has a 145-kDa N-terminal non-collagenous (NC1) domain, an extended central, triple-helical domain, and a short 34-kDa non-collagenous C-terminal (NC2) domain (Fig. 5.3a). The contribution of type VII procollagen to the mechanical stability of the dermal–epidermal junction depends on the ability of single molecules to self-assemble into highly ordered anchoring fibrils. On secretion, some of the C-terminal NC2 domain is proteolytically removed and *cysteine cross-links form triple helical reverse dimers* (Fig. 5.3b). At either end of a dimer, the large NC1 domain can interact with type IV collagen, type I collagen, or laminin-5. The interaction with type I collagen fibers may promote both type VII dimerization and fibril formation. As type VII fibrils form, the stromal ends bind to type IV collagen anchoring plaques deep within the stroma (Fig. 5.1).

Type IV collagen is responsible for the dense network that characterizes the lamina densa and anchoring plaques in all mammals. It is made up from two or three of six homologous α -procollagen type IV polypeptide chains (Fig. 5.4a) that assemble at their C-terminal procollagen domain into one of three heterotrimers called protomers (Fig. 5.4b): $[\alpha_1(\text{IV})]_2[\alpha_2(\text{IV})]$ beneath an epidermis such as the gingival mucosa or skin; $[\alpha_3(\text{IV})][\alpha_4(\text{IV})][\alpha_5(\text{IV})]$ in the glomerulus of the kidney and lungs; and $[\alpha_5(\text{IV})]_2[\alpha_6(\text{IV})]$ around smooth muscle cells. Each individual polypeptide has a long, collagenous domain (gly-X-Y repeats) but frequent interruptions reduce rigidity. The C-terminal non-collagenous region (NC1 region) of the three polypeptides making up each protomer (Fig. 5.4C) links the C-terminal end of each pair of protomers (Fig. 5.4D).

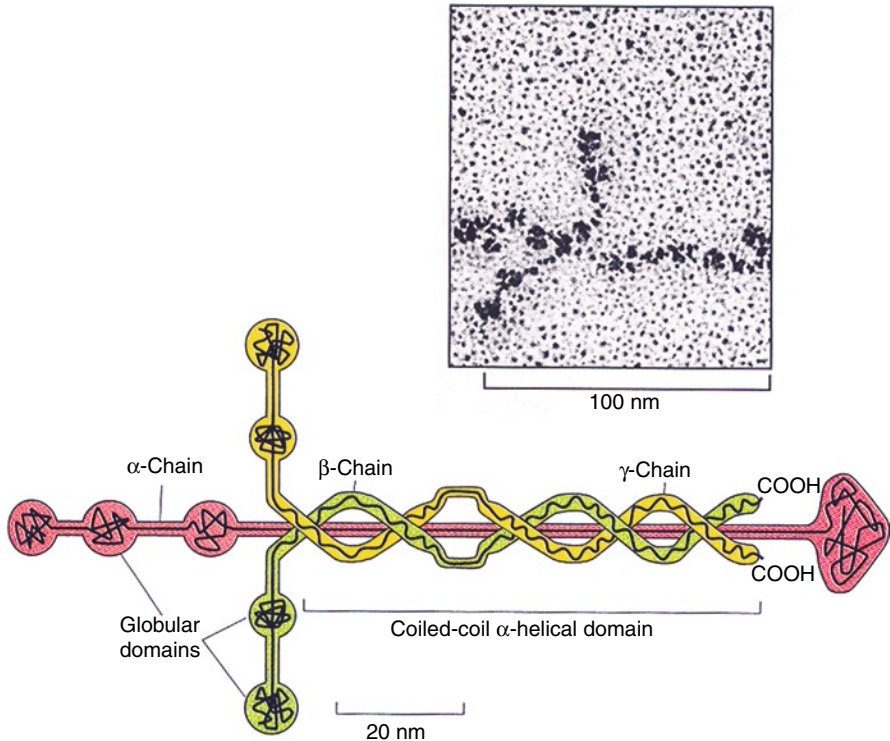
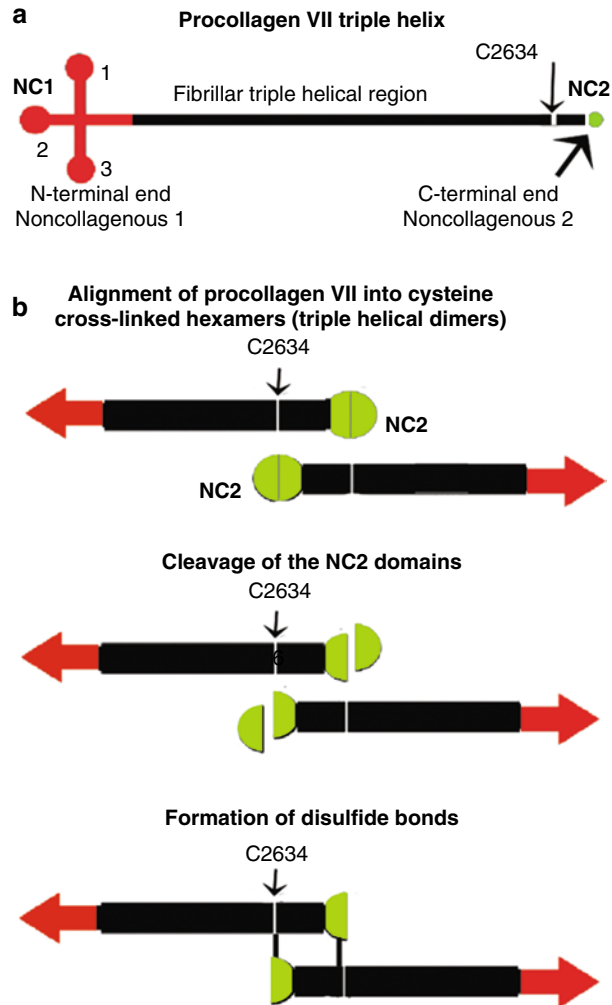


Fig. 5.2 Structure of laminin. Laminin is composed of three homologous polypeptides (α , β , and γ), each more than 1,500 amino acids long. They contain many domains that come together in a central, disulfide-bonded, triple helical coiled-coil with globular ends. Each trimer spontaneously interacts with others to form a web-like polymer illustrated in the electron microscope photomicrograph (*upper right*). Laminin-1 ($\alpha 1$, $\beta 1$, $\gamma 1$) forms an asymmetric cross-linked structure shown on the electron micrographs of laminin shadowed with platinum, whereas laminin-5 ($\alpha 5$, $\beta 1$, $\gamma 1$) forms a filament resembling a thin, barbell rod shown in Fig. 5.6 (*Upper figure*; Reprinted from J. Mol. Biol. Vol. 150, Shapes, domain organizations and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. Engel J, Odermatt E, Engel A, Madri JA, Furthmayr H, Rohde H, and Timpl R. Pages No. 97–120, 1981: with permission from Elsevier. *Lower figure* is adapted from Fig.19-57 in The Molecular Biology of the Cell. B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York)

5.1.2. Hemidesmosomal Proteins

Hemidesmosomes (HDs) are membrane-associated adhesive junctions linked to the filamentous networks of the epithelial cell cytoskeleton and the lamina lucida (light green/dark blue region in Fig. 5.1). The cytoskeleton of all mammalian cells is composed of three kinds of filaments: microfilaments, intermediate filaments and microtubules. Microfilaments

Fig. 5.3 Dimeric structure of collagen type VII anchoring fibrils. **(a) Type VII collagen triple helix.** The non-collagenous (NC) N- and C-terminal procollagen domains (NC1 and NC2 regions) are respectively colored *red* and *green*. **(b) Fibril formation.** Antiparallel binding of two triple helices may be orientated by each helix binding to a type I collagen fiber followed by proteolytic cleavage within the C-terminal non-collagenous region (NC2 region). The cysteine at residue 2634 aligns with a cysteine residue near the cleaved C-terminus so that two triple helical regions are correctly cross-linked to form a hexamer from which a triple helix extends in either direction (Modified from Figs. 1 and 2 in R. Brittingham et al. (2005 Jan 7) "Single amino acid substitutions in procollagen VII affect early stages of assembly of anchoring fibrils." *J. Biol. Chem.* 280(1):191–198)



are thin and made of actin, whereas microtubules are thick and made of tubulin. Intermediate filaments are of intermediate size (~10 nm thick) and they include keratins in epithelial cells (which also secrete them to form hair and nails); nuclear lamins (which form a network that stabilizes the inner membrane of the nuclear envelope); neurofilaments (which strengthen the long axons of neurons); and vimentins (which provide mechanical strength to muscle and other cells). All intermediate filaments possess a pronounced α -helix. Keratins are described in detail in Sect. 5.2.2).

Hemidesmosomes are made up of four proteins: two bullous pemphigoid (BP) antigens, (BP180, an anchoring fibril identified as type XVII collagen, and BP230, a plakin protein), and two other proteins; plectin (a second plakin protein), and integrin $\alpha 6 \beta 4$. BP antigens are proteins that function abnormally in the disease, *epidermolysis bullosa* (Sect. 5.1.3.).

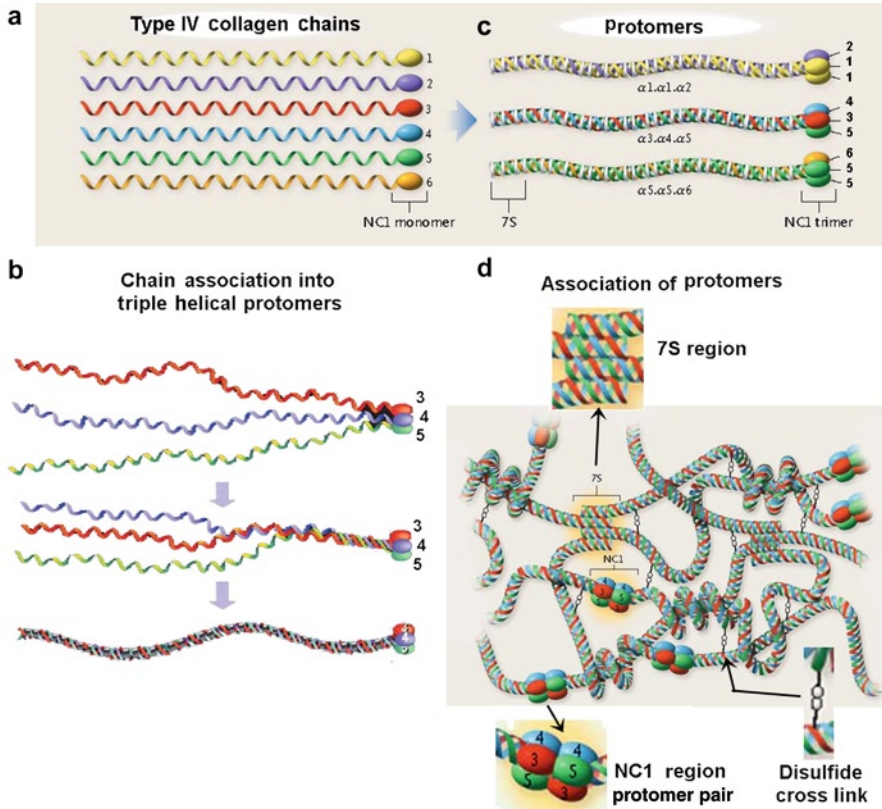


Fig. 5.4 Schematic illustration of type IV collagen supramolecular network assembly. **(a)** *Type IV collagen chains*. There are 6 α -chains (α_{1-6}) encoded in the mammalian genome, each characterized by a long central collagen triple helix, a 7S domain at the N terminus, and a globular, non-collagenous (NC) trimer at the C terminus (NC1). **(b)** *Association into protomers*. The NC1 domain at the C-terminus (Type IV collagen has only one non-collagenous domain) induces triple helix formation from C- to N-terminus in a protomer. The formation of protomer [$\alpha_3, \alpha_4, \alpha_5$] is illustrated. **(c)** *Only a few protomers form*. The ability of only certain 7S or NC1 domains to associate [$(\alpha_1)_2 \alpha_2$], [$\alpha_3 \alpha_4 \alpha_5$] and [$(\alpha_5)_2 \alpha_6$] explains the limited triple-helical associations of the polypeptides. **(d)** *Network formation and cross-linking*. The supramolecular network is assembled by four protomers associating at the non-collagenous 7S domain, followed by dimerization and disulfide cross-linking at the C-terminus. **(b – Slightly modified from Fig. 1B from Borza DB, Hudson BG (2003 May) “Molecular characterization of the target antigens of anti-glomerular basement membrane antibody disease.” Springer Semin Immunopathol. 24(4):345–361); a, c, d – Slightly modified from Hudson BG, Tryggvason K et. al. (2003 Jun 19) “Alport’s Syndrome, Goodpasture Syndrome, and Type IV Collagen.” N. Engl. J. Med. 348(25):2543–2556)**

Plakin proteins link intracellular keratin intermediate filaments to hemidesmosomes of basal epidermal cells, or to desmosomes on suprabasal epidermal cells (Sect. 5.2.1), whereas type XVII collagen (*the only collagen secreted by epithelial cells*) and integrins attach hemidesmosomes to a basal lamina. Three $\beta 1$ integrins (with $\alpha 2$, $\alpha 3$ or $\alpha 5$ partners)

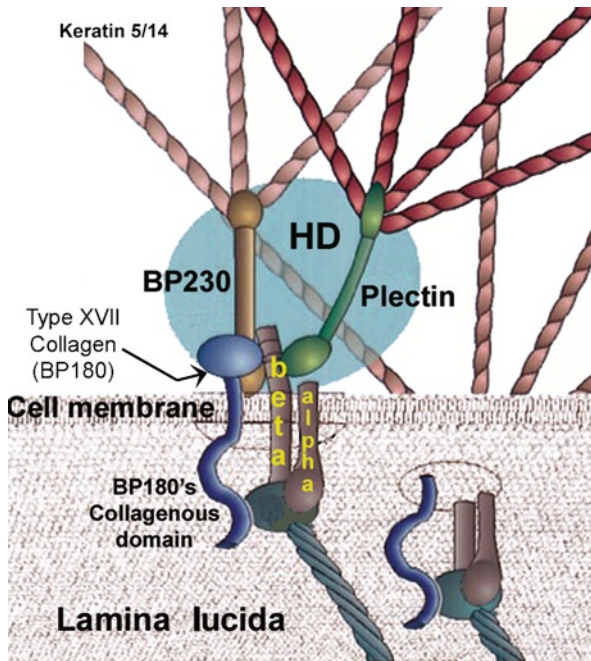


Fig. 5.5 Model of the hemidesmosome (HD)–basal lamina junction. This is an enlarged region of one of the hemidesmosomes pictured in Fig. 5.1. There are 4 components: integrin alpha and beta polypeptides (*dark brown/yellow typescript*), BP180 (type XVII collagen, *dark blue tadpole-shaped*), BP230 (*light brown*) and plectin (*dark green*). They all cluster together around the intracellular side of the integrin beta polypeptide within a hemidesmosome (HD, *blue oval*). The head portion of type XVII collagen (BP180) is attached intracellularly along with BP230 to the β -integrin subunit. The tail portion extends through the cell membrane alongside the β -integrin subunit and together they enfold a laminin-5 head (*dark bluish gray*). The $\beta 1$ integrins which are present in addition to integrin $\alpha 6\beta 4$ bind to many other lamina lucida components besides laminin-5, and initiate keratinocyte differentiation intracellularly (see text). BP230 and plectin are each connected to keratin 5/14 dimers intracellularly and therefore attach the hemidesmosome to keratin intermediate filaments that hold an epithelium together with desmosomes (Sect. 5.2). (Modified from Fig. 8 in Colognato H, Yurchenko PD (2000 Jun) “Form and Function: the Laminin Family of Heterotrimer.” *Dev. Dyn.* 218(2):213–234)

together with integrin $\alpha 6\beta 4$ can attach any of the laminins in the lamina lucida, not just laminin-5. The $\beta 1$ integrins attach laminin-5 by various laminin motifs, not exclusively the arg-gly-asp (RGD) motif described in Sect. 3.2.1.

Type XVII collagen is composed of three identical procollagen polypeptides, each about 1,500 amino acids in length. It has a tadpole-like shape under physiological conditions (deduced from rotary shadowing electron microscopy of bovine cell lines or the pure protein). The protein is one of very few proteins whose N- and C-termini are *inverted with respect to the membrane*. In nearly all transmembrane proteins, for example integrins, the C-terminus is cytosolic and the N-terminus is extracellular. In type XVII collagen, the cytosolic N-terminal domain comprises about a third of the amino acid residues

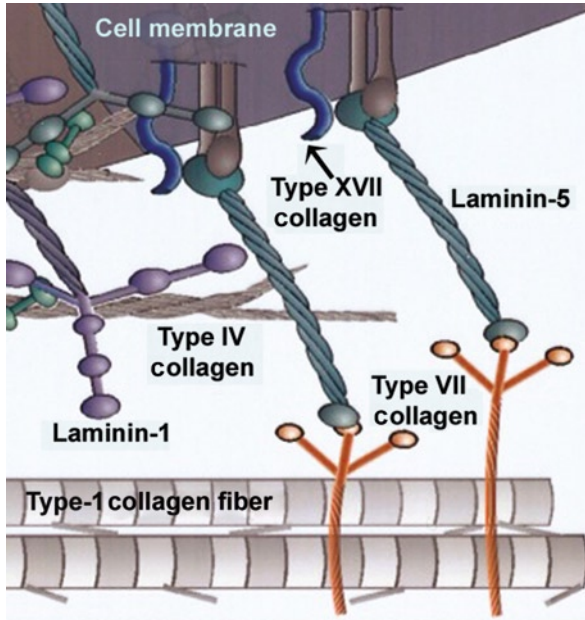


Fig. 5.6 Basal lamina anchoring to the dermis. The long filament of laminin-5 (blue gray) attached to integrin and type XVII collagen beneath the hemidesmosome (HD) (*top right*) protrudes through the type IV collagen network (*brown fibers*) to attach type VII anchoring collagen (*dark orange fibers* with three heads corresponding to each constituent α_1 -procollagen polypeptides; *bottom right*). Type VII collagen forms dimers (Fig. 5.3) that loop around type I collagen fibers to the anchoring plaques (Modified from Fig. 8 in Colognato H, Yurchenko PD (2000 Jun) "Form and Function: the Laminin Family of Heterotrimer." *Dev. Dyn.* 218(2):213–234)

and ends in a short, trans-membrane hydrophobic sequence. The C-terminal extracellular domain is a long, interrupted Gly-Pro-X sequence which forms a flexible triple helix like type IV collagen and anchors itself to the head region of a laminin-5 filament (Fig. 5.6), supplementing the integrin connection and strengthening the basal cell attachment to the lamina lucida by hemidesmosomes. At their stromal end, the laminin-5 fibrils are anchored to type VII anchoring collagen fibrils (see Sect. 5.1.1). The epidermis may be pulled away from the dermis at the lamina lucida, which does not have the strength of collagen. This weakness may also allow the epidermis to repair or grow along with changes to the dermis as dictated by development or the environment.

5.1.3. Basal Lamina of the Dental Epithelial Attachment

At the base of a gingival sulcus, the junctional epithelium mediates the epithelial attachment. This epithelium possesses two basal laminas, an outer one that is continuous with the sulcular epithelium around the free gingiva and an inner one which mediates the actual

dental epithelial attachment (Fig. 5.7a). The inner basal lamina contains only keratinocyte-secreted products: type XVII collagen, laminin-5, and integrins. Laminin-1 and type IV collagen, the fibroblast-secreted components, are absent (Fig. 5.7b). Teeth movements continuously alter the junctional epithelial cell environment, causing the integrins and type XVII collagen to release and reattach laminin-5 to the tooth surface (Fig. 13.1).

Epidermolysis bullosa (EB) is caused by mutations that affect epidermal basal cells, the basal lamina that they synthesize, or fibroblast cell products with which they interact. A mutation that causes EB may affect any one of the following: epithelial basal cell keratins (Sect. 5.2.2), plectin, integrin $\alpha_6\beta_4$, type XVII collagen, laminin-5, or type VII collagen. The disease manifests as a blistering of the skin and mucous membranes at the dermal-epidermal junction. A similar aberrant interaction prevents enamel-forming cells (*ameloblasts*) from aligning correctly against calcified collagen fibrils of dentin, the template for enamel matrix calcification (Sect. 9.5.1). The enamel does not properly calcify (hypoplastic enamel), and the affected individual becomes prone to severe dental caries. These latter individuals usually present with a form of EB that is classified as *junctional epithelial bullosa* in which the blistering is due to mutations of a structural component: integrin $\alpha_6\beta_4$, type XVII collagen or laminin-5 (Table 7.1).

In addition to integrin $\alpha_6\beta_4$, epidermal basal cells, which include junctional epithelial cells, possess two $\beta 1$ integrins that affect subsequent basal cell differentiation (Sect. 5.2.2). The $\beta 1$ integrins transmit inside-out signaling via kindlin-1, a protein that binds to the

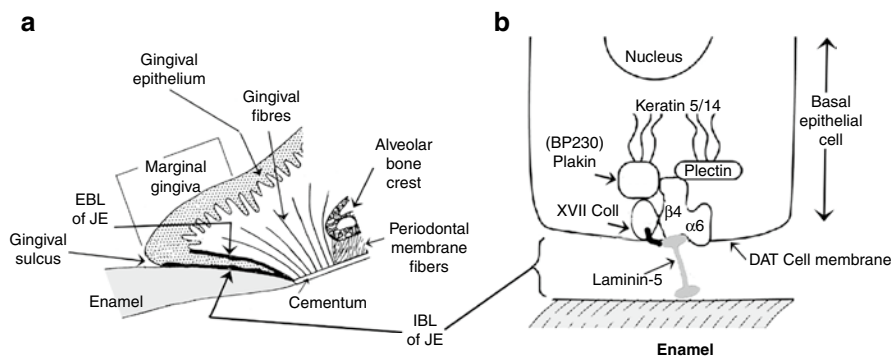


Fig. 5.7 Composition of the gingival epithelial attachment's basal lamina. **(a)** *Gingival region rotated anti-clockwise* – See Fig. 12.1 for an un-rotated view. **(b)** *Components of the internal basal lamina of junctional epithelium (IBL of JE)* Dentally attached (DAT) cells make hemidesmosomes which form the internal basal lamina of junctional epithelium (IBL of JE). The DAT cell intracellular hemidesmosome connections are as shown in Fig. 5.5, but its internal basal lamina (IBL) is composed only of a thin layer of laminin-5 polymers attached to type XVII collagen and integrin $\alpha_6\beta_4$ at one end, and to tooth enamel at the other. Laminin-1 and type IV collagen are absent from the IBL because there are no fibroblasts to make them. The external basal lamina of junctional epithelium (EBL of JE) contacts the gingival connective tissue stroma as shown for the epithelial-connective tissue basal lamina in Figs 5.1, 5.5, and 5.6 (Slightly modified and updated version of Fig. 5 in Pollanen MT, Salonen JJ, and Uitto V-J. Structure and function of the tooth–epithelial interface in health and disease, *Periodontology* 2000, 31:12–31, 2002. With copyright permission from Wiley-Blackwell, PO Box 805, 9600 Garsington Road, Oxford OX4 2DQ, UK)

integrin cytoplasmic domain. Mutations of kindlin-1 upset the differentiation of epidermal basal cells and give rise to a form of EB, *Kindler syndrome* accompanied by aggressive periodontitis (Sect. 14.1.1). In other forms of EB, the absence of a fibroblast interaction may protect the junctional epithelial attachment from developing aggressive periodontitis.

Basal laminas are made up of type IV collagen and laminin, each a trimer of separately encoded polypeptides. The outer epithelial side (lamina lucida), is composed of laminin-1 filaments and laminin-5 filaments. Laminin-5 is attached to hemidesmosomes by integrin $\alpha 6 \beta 4$ and type XVII anchoring collagen (bullous pemphigoid antigen-180, BP180). The latter is a transmembrane protein whose non-collagenous N-terminal head lies in the cytosol. The head is linked to intermediate filaments (keratins) by the cytoplasmic domain of integrin $\beta 4$ via two proteins (plectin and BP230). The fibroblasts on the dermal (stromal) side secrete a lamina densa, mostly laminin-1 and type IV collagen. The latter is tightly attached to the dermis by anchoring collagen fibrils (type VII) which attach type IV collagen to type I collagen fibers and other stromal proteins. Epidermolysis bullosa (EB) is caused by a mutation that alters laminar structure, or dermal or hemidesmosomal attachments. It is manifest as blistering within the basal lamina. A variant, junctional EB, is due to mutations of type XVII collagen or laminin-5 which additionally interfere with ameloblast/odontoblast interactions. Another variant, Kindler syndrome, is the only form of EB accompanied by aggressive periodontitis. This EB variant is due to a mutation in kindlin-1, an intracellular protein that provides an inside-out signal for $\beta 1$ integrin-mediated control of epidermal cell differentiation.

5.2.1.

General Structure of Skin, Oral and Junctional Epithelia

An epithelium (plural: epithelia) is classified as *simple*, *stratified* or *transitional* (Fig. 5.8a). A *simple epithelium* consists of a single layer of continually-dividing cells that mediate an exchange of metabolites between compartments. Capillary, kidney tubular, and intestinal epithelia are examples of simple epithelia. They respectively exchange metabolites in interstitial fluid (Chapter 3, section 3.3.1.), between the glomerular filtrate and blood plasma (Chapter 10, section 10.3.1.), or pass mono- and di-saccharides, amino acids and fatty acids from digested food to blood plasma. By contrast, a *stratified epithelium* such as that of the skin is layered, and designed to prevent fluid diffusion and metabolite exchange. A *transitional epithelium* is a layered epithelium that becomes simple when the tissue is stretched, for example the bladder epithelium.

A basal lamina is permeable to interstitial fluid which provides the nutrients for the *basal layer* of cells to proliferate. As discussed in Sect. 5.1, basal cells adhere to the basal lamina using hemidesmosomes. In simple epithelia, basal cells either divide into identical daughter cells or undergo apoptosis (programmed cell death, Sect. 13.4.1). In a stratified

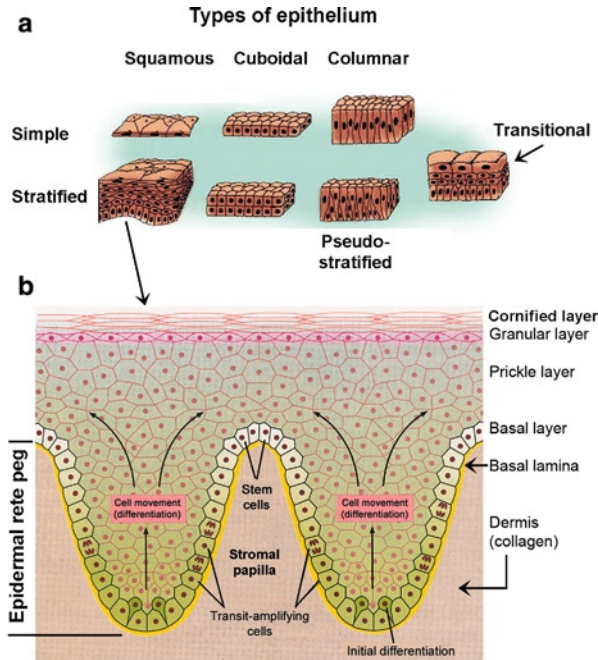


Fig. 5.8 Skin epithelium. (a) *Types of epithelium.* Epithelia are primarily classified as simple or stratified and secondarily as squamous, cuboidal, or columnar. A transitional epithelium is neither simple nor stratified. (b) *The four epithelial cell layers of a fully stratified epithelium.* Names of the layers are indicated on the right. The basal cell layer consists of undifferentiated (stem) cells between the rete pegs and committed, actively dividing (transit-amplifying) cells along the length of the rete pegs. The transit-amplifying cells move down, toward the tip of a rete peg where they start to differentiate. Each stromal papilla surrounding the basal and transit-amplifying cells is rich in collagen and capillaries (Slightly modified from Fig. 22-6 in *The Molecular Biology of the Cell*, B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York)

epithelium, the basal cells are a mixture of undifferentiated *stem cells* and *transit-amplifying cells*. The former divide slowly and remain strongly attached to the basal lamina (Fig. 5.8b), whereas the latter are stem cell progeny that differentiate into a layer next to the basal layer (Fig. 5.8b). Until they move out of the basal layer, they divide faster than stem cells, creating finger-like projections (rete pegs) that bulge into the stroma.

Differentiation starts when the transit-amplifying cells auto-digest their hemidesmosomes and synthesize additional *desmosomes*, which appear as thick tufts resembling ‘microscopic prickles’ on their outer surface. As they are pushed further away by the continuing basal cell division, the *prickle cells* synthesize different keratins (Sect. 5.2.2) whose strong desmosomal attachment causes the cells to flatten and elongate. Eventually, a thin, dark-staining region develops, the *granular layer*, in which cells contain brown granules of keratin precursors called *hyalokeratin*. Desmosomes mediate mechanical cohesion and fluid permeability in the basal and prickle layers, but the granular layer is fluid-impermeable. The flattened cells of the granular layer undergo apoptosis (Chapter 13, section 13.4.1.),

losing their internal cytosolic structures and becoming *scales* or *squames*, i.e. cell membranes filled only with keratin. Unlike skin, the cells of the outer surface of the hard palate and gingival mucosa retain their nuclei and are said to be *parakeratinized*, not fully keratinized (*orthokeratinized*) like the skin.

5.2.2.

Composition of Desmosomes and Keratins

Desmosomes (Fig. 5.9) are *punctate adhesions* between adjacent keratinocyte cell membranes. These membranes are unusually thick and are referred to as *plaques*, thick membrane discs connected by desmosomal proteins that anchor adjacent keratinocytes and they provide strong intercellular cohesion. The *desmosomal plaques* should not be confused with *bacterial plaques* or *biofilms* (Chapter 1, section 1.3.2.). The desmosomal proteins anchor adjacent cells and provide proximal cohesion. Desmosomes are composed mostly of two different types of proteins: *cadherins* and *plakins*. The cadherins, mostly *desmoglein* and *desmocollin*, are transmembrane proteins that provide intermembrane attachments by forming heterodimers. On the cytosolic side of the membrane, cadherins are attached to plakin proteins that link desmosomes to the intracellular keratin filaments just as they link hemidesmosomes in the basal cell layer. In the outer layers of the epithelium where mechanical stress is important, the types of desmogleins and desmocollins change and the content of *desmoplakin* increases. There are other, less prominent types of junctions between keratinocytes, primarily the *adherens* and *tight junctions*. Small numbers of these other junctions are critical for maintaining epidermal health and the fluid barrier.

The keratin family of proteins comprises two of the six classes of intermediate filament proteins found in the cytosol of eukaryotic cells. They provide the *intracellular* structural stability that complements the *intercellular* mechanical cohesion provided by desmosomes. Keratin filaments cross the entire cytosol of keratinocytes and their ends are tightly attached to desmosomes by desmoplakin (Fig. 5.9) or, in basal cells, to the related plakin (BP230) and plectin proteins in hemidesmosomes.

Note: Keratin is an intracellular filamentous protein whereas keratan is a sulfated polysaccharide found extracellularly in cartilage and dermal (stromal) connective tissue.

Keratin polypeptides are encoded as two classes: type I, acidic (K9 through K19) and type II, basic (K1 through K8). Both classes form extended α -helical domains. Keratin filaments are composed of heterodimers: type I keratin α -helices supercoiled around type II keratin α -helices in a (left-handed) coiled-coil (Fig. 5.10). Monomeric heterodimers become cross-linked to each other by disulfide bonds and then assemble rapidly and spontaneously into 100 Å diameter filaments in staggered arrays as in fibrillar collagen (Sect. 4.1.1). The basal cells express K5 and K14, which change to K1 and K10 as they differentiate into prickly cells. The K1–K10 heterodimer provides structural stability and rigidity for the skin, hard palate, and buccal surface of the free and attached gingiva. The

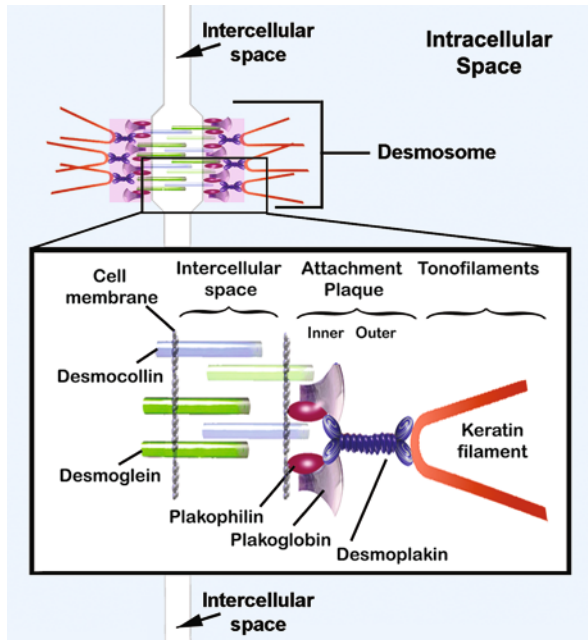


Fig. 5.9 Molecular composition of a desmosome. The upper portion of the figure shows the general outline of a desmosome crossing the intercellular space between keratinocytes and its attachment plaque consisting of plakin proteins attached to the respective inner cell membranes. The lower half is a blow-up of part of that region to show the protein structures and interactions. Transmembrane cadherin proteins, desmocollin (blue) and desmoglein (green) form an extended chain heterodimer that links the plakin proteins on adjacent cells. On the inner phospholipid layer, desmocollin is attached to plakophilin and desmoglein to plakoglobin. In turn plakoglobin and plakophilin are attached to one end of desmoplakin whose other end is attached to the keratin intermediate filament. Desmoplakin therefore mediates the attachment of the cytoskeleton to the desmosomes which increases as the cells differentiate (see text) (Original figure by Dr Wirsig-Wiechmann)

gingiva also contains the K6/K16 pair (Fig. 5.11a). Besides K5 and K14, the basal layer of all squamous epithelia expresses *three integrins* ($\alpha 2\beta 1$, $\alpha 3\beta 1$, and $\alpha 6\beta 4$) on their outer cell surface. The two $\beta 1$ integrins interact with kindlin-1 to control the differentiation of basal cells to prickle cells (Sect. 5.1.3).

5.2.3. Oral and Junctional Epithelium

The nonkeratinized regions of the oral mucosa, the mobile mucosa of the cheeks, lips, ventral surface of the tongue, soft palate and the oral sulcular and junctional epithelia are permeable to fluids and small molecules (Sect. 5.2.1). The prickle cell layer of these epithelia expresses mostly K4 and K13 instead of K1 and K10.

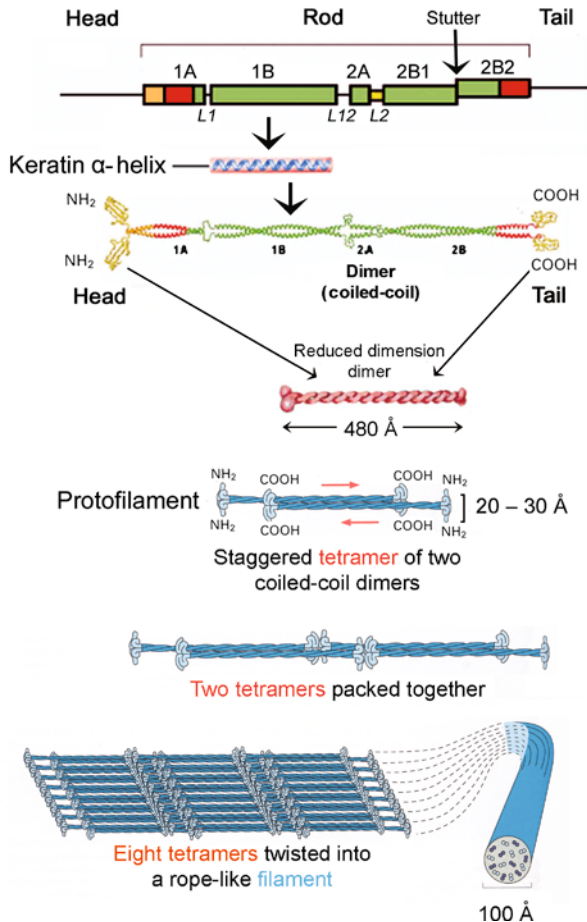


Fig. 5.10 Keratin structure. The protein is almost entirely composed of α -helical rod domains (orange, red, and green boxes) linked by non-helical linker regions. The latter are indicated as two internal lines and a thin yellow square. The N-terminal domain (head) forms a β -sheet and the C-terminal domain (tail) has a complex tertiary structure. A typical keratin α -helix domain (1B) is indicated along with its position with other helical domains and the N- and C-terminal domains in a dimer. Two dimers assemble alongside each other staggered in opposite directions to form a tetramer protofilament. Eight tetramers then assemble to form a filament (bottom) (Figure is a pastiche. Top two rows are from Fig. 1A and B from Kirfel J, et al. (2003 Jan) "Keratins: A Structural Scaffold with Emerging Functions." *Cell. Mol. Life Sci.* 60(1):56–71. These are interspersed with the top two lines of Fig. 4-11 from Lehninger Principles of Biochemistry. D.L. Nelson and M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., New York; the remainder is from Fig. 16-16 (C through E) in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York)

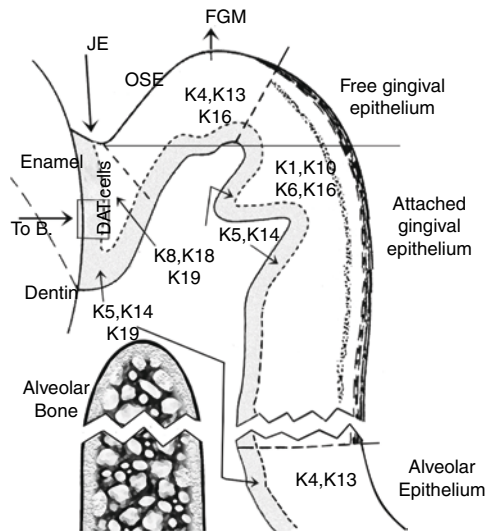


Fig. 5.11 Keratin composition of junctional and gingival epithelia. The free, attached and alveolar epithelia are not drawn to scale. The thicker surface and internal broken lines indicate a cornified keratinized layer that characterizes the epithelium of the free and attached gingiva. In the junctional epithelium (JE) and oral sulcular epithelium (OSE) to just past the free gingival margin (FGM), and also in the alveolar epithelium, the prickle cells possess K1 and K10 instead of K1 and K10. These epithelia are non-keratinized; the granular and cornified layers are absent. K16 is present in the oral sulcular epithelium without a partner and its function there is not known. K19 pairs with any type II keratin. (Slightly modified by Dr. Wirsig-Weichmann from Fig. 5 in Pollanen MT, Salonen JI and Uitto V-J, Structure and function of the tooth-epithelial interface in health and disease. Periodontology 2000, 31:12–31, 2003)

DNA for genetic analysis is often obtained from the nuclei of nonkeratinized mucosal cells from inside the cheek. Collecting these cells is less invasive and simpler than obtaining a sample of blood cells.

Junctional epithelium seals the periodontium from the oral cavity at the base of a gingival sulcus and it is often referred to as the junctional epithelial attachment or just the epithelial attachment (Fig. 5.12a). Hubert Schroeder and Max Listgarten published the first comprehensive description of this epithelium in a monograph in 1973. The junctional epithelium develops at the base of a sulcus where the reduced enamel epithelium (Sect. 9.5.1) merges with the apical end of the oral sulcular epithelium (OSE) (Fig. 5.12b). It may also develop from regenerating oral sulcular epithelium if the sulcus is surgically excised by a procedure called *gingivectomy*. The *external* (outer) epithelial basal layer forms a basal lamina that contacts the gingival stroma and is continuous with the basal layer and lamina of the oral sulcular epithelium. At its apical extremity (only a few cells thick), the external basal cell layer and lamina unite with an *internal* (inner) basal layer of cells whose lamina is dentally attached and extends coronally to slightly above the base of the gingival sulcus (Fig. 5.12b).

The biochemistry and metabolism of junctional epithelium has been studied by many investigators, but most thoroughly by Ian Mackenzie and Jukka Salonen. *Both junctional basal layers are rapidly proliferating, transit-amplifying cells.* They are derived from stem cells located where the external basal layer meets the basal layer of oral sulcular epithelium (Fig. 5.12a). Because basal cells must proliferate to produce a basal lamina and remain attached to an underlying surface, interstitial fluid must penetrate the whole junctional epithelium to the tooth surface to maintain internal basal cell proliferation and attachment. Junctional epithelium is therefore permeable to stromal fluid and in this respect it resembles a simple epithelium. *Interstitial fluid transudes throughout the junctional epithelium*

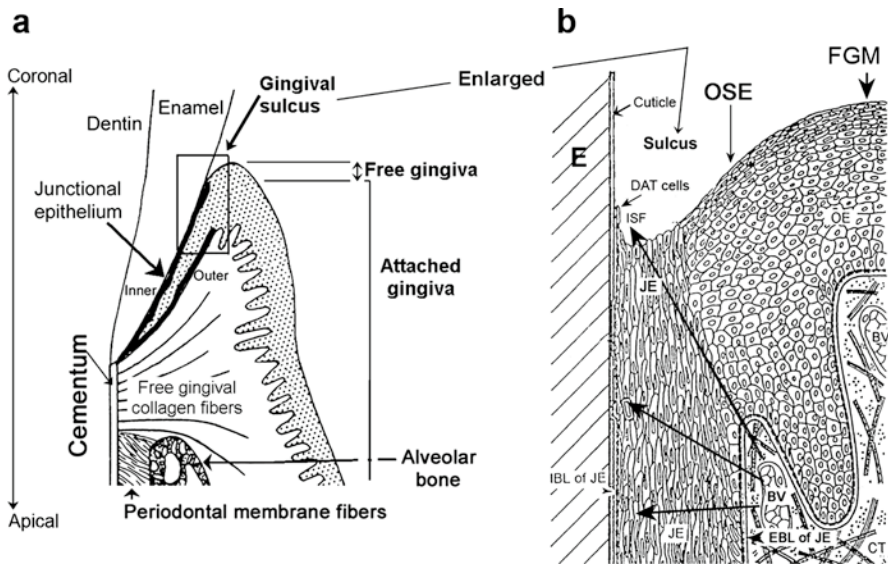


Fig. 5.12 Coronal extremity of the periodontium showing the gingiva. **(a)** *The gingival region.* The oral sulcular epithelium and oral epithelium below the free gingival margin (FGM) comprise the *free gingiva* which is tightly held against the tooth by free collagen fibers. The *attached gingiva* (length slightly exaggerated) is attached to the alveolar bone by collagen fibers. The junctional epithelial attachment has outer and inner basal cells (*thick black line*) that merge apically. The outer basal cells merge coronally with the basal cells at the apical end of the oral sulcular epithelium and the site of junctional epithelial stem cells **(b)** *The gingival sulcus.* The gingival sulcus (Sulcus) is bounded by the enamel cuticle (Cuticle) and oral sulcular epithelium (OSE) which terminates in the free gingival margin (FGM) coronally. Immediately beneath the sulcus, interstitial fluid (ISF) leaks from capillary blood vessels (BV) in the stroma and transudes through the junctional epithelium to provide nutrients for the proliferative dentally attached cells (DAT cells), at the base of the sulcus (*long arrow*) and apically (*two short arrows*). The DAT cells lie against the inner basal lamina (IBL) and the similarly proliferative basal cells continuous with those of oral sulcular epithelium lie against the external basal lamina (EBL). This area is enlarged from the box around the gingival sulcus in **a**. Opposite (Modified from Fig. 1-2 in Contemporary Periodontics, edited by RJ Genco, HM Goldman and DW. Cohen. Chapter 1: The Gingiva, Structure and Function by H Loe., MA Listgarten & VP Terranova. Pub., The CV Mosby Co., St Louis MO.) **b** – Modified from Fig. 36A in Schroeder, H.E. & Listgarten, M.A. (1977) Monographs in Developmental Biology, Vol. 2, Fine Structure of the Developing Epithelial Attachment of Human Teeth. Series editor, A. Wolsky, Vol 2, 2nd Ed. Pub., S. Karger, Basel, Switzerland)

from capillaries beneath the external basal lamina (EBL). Traces of this fluid reach the most coronal region of dentally attached cells by passing through the base of the gingival sulcus (Fig. 5.12a).

Basal cells that stop dividing also stop secreting a basal lamina and lose their attachment. They are squeezed by adjacent, dividing basal cells into the body of the junctional epithelium and are expelled into the base of a sulcus. The few tight junctions in the body of the junctional epithelium are consistent with its permeability to interstitial fluid. The rapidly dividing cells of both internal and external basal layers express K5 and K14 like all stratified epithelial basal cells, but the interior cells express K4 and K13, not K1 and K10 (Fig. 5.11). The composition of the internal basal lamina is described in Fig. 5.7b.

The junctional epithelium is continually regenerating itself from stem cells at the union of internal and external basal lamina. It expresses a fourth integrin, $\alpha\beta6$ in addition to the two $\beta1$ integrins and $\alpha4\beta6$ integrin. Integrin $\alpha\beta6$ is a marker for newly developing or regenerating epithelia and it binds to the RGD sequence of laminin-5 (Sect. 5.1.2). A regulatory protein, TGF- $\beta1$ (Section 3.2.2.) is continually secreted by junctional epithelium and it binds to integrin $\alpha\beta6$. The binding exposes TGF- $\beta1$ to proteases that hydrolyze off a *latent activation peptide* (LAP), providing mature (processed) TGF- $\beta1$ in the inner and outer basal laminae. Among its many functions, mature TGF- $\beta1$ inhibits inflammatory responses. Thus, the activation of TGF- $\beta1$ by integrin $\alpha\beta6$ in the junctional epithelium inhibits a potential inflammatory response to masticatory tooth movements that, uncontrolled, could result in spontaneous periodontitis. The junctional epithelium is the initial target of bacteria that initiate gingivitis and is eventually destroyed (Chapter 13, section 13.3.1.).

Epithelial cells contain keratins: cytosolic, intermediate-sized filaments. Each acidic keratin (type I) is partnered with a basic keratin (type II) to form extended, intertwined alpha helices. These heterodimers extend longitudinally and laterally in staggered and/or end-to-end arrays to form cytosolic intermediate filaments. Different keratin types account for the properties of different types of epithelia. A stratified epithelium provides a barrier consisting of multiple layers of cells in which the basal layer expresses K5–K14 but the supra-basal layers express K1–K10. Junctional epithelium has inner and outer basal layers that contain K5–K14. Its supra-basal layers possess K4 and K13, not K1 and K10 and it therefore has no granular layer. Desmosomes permit water and metabolite exchange beneath the granular layer, but they are mostly absent from junctional epithelium which is fluid-permeable like a simple epithelium. The cells of both basal layers of junctional epithelium proliferate and shed their progeny into the base of the sulcus. Junctional epithelium also secretes and activates TGF- $\beta1$, which prevents an inflammatory response to masticatory trauma.

Extracellular matrix (stroma) contains elastic fibers and glycosaminoglycans (GAGs) that have long been identified histologically. Fibrillin is a major component of microfibrils that surround elastin in elastic tissues and, along with collagen, is an important component of the periodontal ligament. Section 1 describes how fibrillin was isolated, major features of its structure, and mutations that affect the face and oral cavity. Section 2 describes how elastin was isolated, the major features of its structure, the importance of copper in elastin processing and its contribution to stromal flexibility. Section 3 describes stromal glycosaminoglycan composition and synthesis. Section 4 describes the glycosaminoglycan proteins and their association with type II collagen in cartilage. The chapter concludes with a discussion of the structure and function of all collagen-glycosaminoglycan-associated proteins (Sect. 5).

6.1.1. Fibrillin

When tissues containing elastic fibers are extracted with 6.0 M guanidine buffers, a large molecular weight aggregate appears with a characteristic beads-on-a-string structure. The major component of this structure is a ~350 kDa glycoprotein identified as fibrillin. Using gold-conjugated antibodies, fibrillin was located to microfibrils with or without elastin. Fibrillin is extremely long, 2,871 amino acid residues in humans. The beaded regions bind calcium ions and have a periodicity of 56 nm, whereas the periodicity of collagen fibrils and microfibrils (types I and VI collagen) is 64 and 3 nm, respectively.

Fibrillin-1 is made up of numerous *calcium-binding* (*cb*) domains, all homologous to the sequence of *epidermal growth factor* (*cbEGF* domains, Fig. 6.1). Each *cbEGF* domain is a beta-sheet held together by cysteine disulfide bonds. In addition, calcium ions bind to aspartate and glutamate residues at the N-terminus of each *cbEGF* domain (Fig. 6.1a). *EGF* domains occur in diverse membrane-bound or secreted animal proteins, but usually without the calcium-binding sub-domain which is a separate N-terminal domain in *EGF* itself. The entire fibrillin-1 molecule consists of 43 *cbEGF* domains (shown in blue in Fig. 6.1b). Short *linker domains* between the *cbEGF* (green ovals in Fig. 6.1b) are homologous to domains in proteins that bind transforming growth factor- β 1 (*TB* domains). As discussed

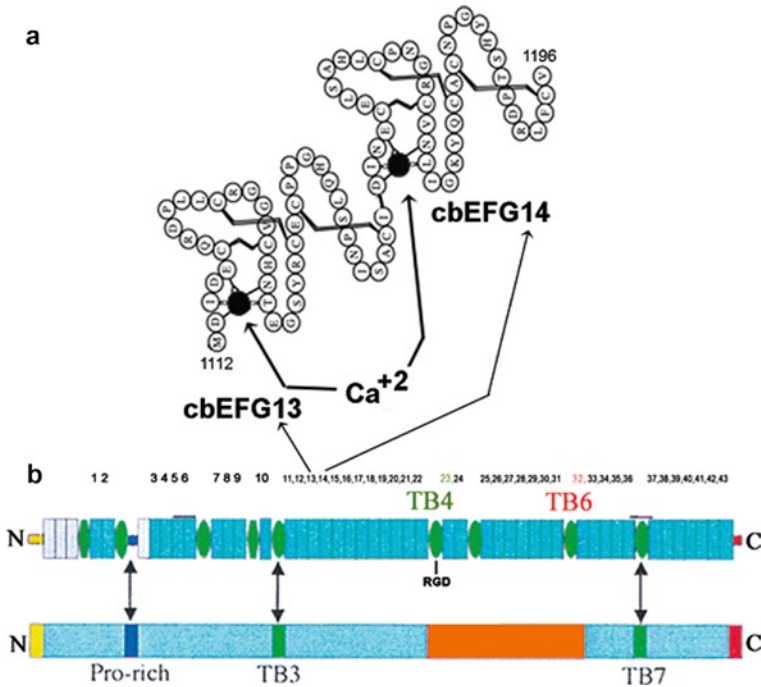


Fig. 6.1 Fibrillin individual domains. **(a)** Detailed structure of the fibrillin cbEGF13–14 pair. The amino acids are numbered from N- to C-terminus. The bound calcium ions (one per cbEGF domain) are indicated as black circles close to the N-terminus of each domain. The cysteine cross-links (three per cbEGF domain) are indicated by zigzag lines. **(b)** Fibrillin domain structure. The 43 cbEGF domains (blue) are numbered. There are four additional more complete epidermal growth factor (EGF) domains at the N-terminal region (gray). There are nine TB domains, but only seven are linker domains (green ovals). The TB1 domain is linked to a separate proline-rich domain (dark blue) and TB3 (linked to cbEGF10) is modified (green diamond). The TB linker domains are a potential source of flexibility, through their interaction with their flanking cbEGF domains. The interbead segment, TB4–6 and intervening cbEGF domains (orange segment at the foot of Fig. 4.17b) are the compressed, normal form that expands under tension by releasing Ca^{2+} to give the 70-nm beaded form (**a** – From Fig. 3 in Whiteman P. et al. (1998 Apr 3) “A Gly $\rightarrow$ Ser change causes defective folding in vitro of calcium-binding epidermal growth factor-like domains from factor IX and fibrillin-1.” J. Biol. Chem. 273(14):7807–7813. **b** – Reprinted with minor changes from Arnaout MA (2004) “The structural basis of elasticity in fibrillin-based microfibrils.” Structure 12(4):734–736; Copyright 2004, with permission from Elsevier)

previously (Sect. 3.2.2), *latent transforming growth factor- β* (latent TGF- β) binds to thrombospondin-1 (TSP-1) by its latent activated peptide (LAP) domain, exposing the potentially active C-terminal domain to be released by stromal protease cleavage. An alternative activation mechanism exists in which the latent TGF- β binds instead to fibrillin at TB-cbEGF hybrid domains near the N terminus (Narrow green ovals on the N-terminal side of cbEGF domains 1 and 10 in Fig. 6.1b). Fibrillin also binds *bone morphogenic proteins* (BMPs) at this site. BMPs other than BMP-1 which is a zincin protease (Sect. 8.2.1) are

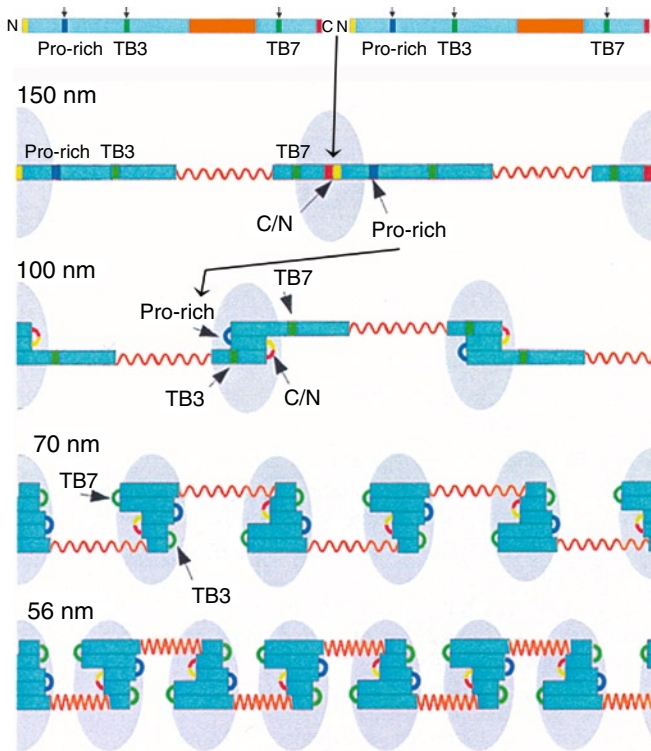
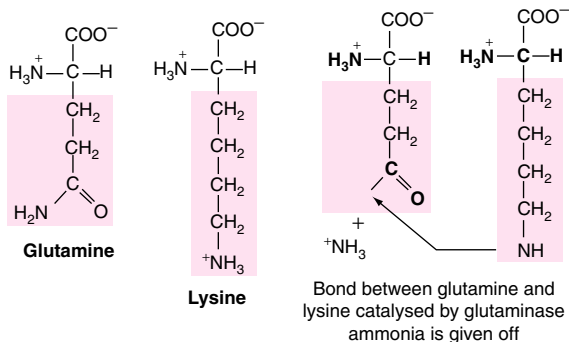


Fig. 6.2 The hinged model of fibrillin elasticity. Fibrillin forms dimers by covalent N- and C-terminal binding and this permits folding initiation to the 150-nm periodicity. As folding then proceeds to the 100-nm periodicity, glutaminase attaches and forms glutamine-lysine intra-molecular cross-links between the folded segments (*blue*). As folding proceeds further, the fibrillin polypeptide dimer bends at the TB3 and TB7 regions and additional proteins attach to this region. The N and C termini are in *yellow* and *red*, respectively. The interbead region between TB4 and TB6 is elastic. Its β -sheet structure loses calcium ions under tension, allowing disulfide-bonded cysteine residues to move by rotating away from each other (shown in *orange*). Numbers on the left indicate the periodicity. Details of folding are described in the text (Reprinted with minor changes from Structure, Vol 12 (4), Arnaout, MA, The structural basis of elasticity in fibrillin-based microfibrils, pp. 734–736; Copyright 2004, with permission from Elsevier)

homologous to TGF- β . A fibrillin microfibril that binds a BMP (not BMP-1) along with latent TGF- β causes the BMP prodomain to promote TGF- β activation, presumably by a stromal protease like TSP-1 activation. The active disulfide peptide dimer is released and induces osteoblast differentiation and activation for bone development.

Fibrillin forms head-to-tail polymers that progressively fold at defined sites between successive molecules (Fig. 6.2). Initial internal folding at the head-tail junction (red/yellow) produces a 150-nm periodicity. Further folding at the proline-rich region (dark green) produces an approximately 100-nm bead periodicity and creates cross-links (not shown) between the folded regions. These cross-links are mediated by the enzyme transglutaminase, which replaces the terminal amide of glutamine with the ϵ -amino group of an

Fig. 6.3 The transglutaminase reaction. Folding brings the glutamine and lysine residues into close proximity where bound glutaminase replaces the glutamine amide group by linking it to the ϵ -amino group of lysine. The amide is given off as ammonia



adjacent lysine residue, creating an isopeptide, intermolecular cross-link (Fig. 6.3). Transglutaminase also acts on various other proteins, most notably fibrinogen (Sect. 11.3.4). Transglutaminase requires calcium ions for activity and it attaches to folded (beaded) regions (light blue, egg-shaped region in Fig. 6.2) along with other proteins.

The extended central regions of the fibrillin polymer (orange in Fig. 6.2) remain free of associated proteins. Further intramolecular folding at the TB7- and TB3-cbEGF linker regions result in a ~ 70 -nm bead periodicity which corresponds to a “stretched” form. The interbead segment (cbEGF domains from TB4 to TB-6; orange in Fig. 6.2) spontaneously bind calcium ions, which compresses them further to give the observed 56-nm beaded periodicity, “relaxed” form. Stretching occurs within this segment and is reversible. On stretching, fibrillin periodicity increases from 56 to 70 nm due to the interbead segment dissociating bound calcium ions. When the stretching force is released, calcium ions can bind again and this pulls the segment back to the 56-nm “relaxed” form. The folded beaded region normally stays intact, but severe stretching that partially unfolds the beaded regions to above 100 nm prevents the beads from returning to the “relaxed” form (overstretched). Along with elastin (Sect. 6.2.1), fibrillin is an important component of ligaments (Sect. 3.1.3). If a ligament is overstretched it is the fibrillin microfibrils that are damaged and take a long time to be repaired.

Fibrillin-2 has an amino acid sequence that is 68% identical to fibrillin-1 and is coexpressed with fibrillin-1 in many tissues early in mammalian development. It forms head-to-tail fibrillin1/2 alternating heterodimers that resemble fibrillin-1 homodimers shown at the top of Fig. 6.3. During mammalian development, some tissues express fibrillin-2 without fibrillin-1 and fibrillin-2 homodimers may assemble by a mechanism that does not involve fibrillin-1 but perhaps utilizing fibrillin-3, a third member of the fibrillin family. Fibrillin-2 binds to the precursor of elastin during development and forms stronger elastic fibers than fibrillin-1. Fibrillin-3 is a minor component whose functions are uncertain.

Mutations of fibrillin-1 and -2 disrupt elastic tissue scaffolding, particularly in the aorta, eyes, and skin. An especially obvious effect of certain fibrillin-1 and -2 mutations is an overgrowth of the long bones of the body, resulting in long limbs and a tall stature (Marfan’s syndrome). In addition, there are major changes to the face, oral cavity, and teeth, most notably, a highly arched palate, crowded incompletely developed (hypoplastic) teeth and deformities of the roots. These changes may all stem from those fibrillin mutations that

Fibrillin is the major component of beaded microfilaments possessing elasticity. It is secreted as a 150 nm polypeptide possessing almost 50 calcium-binding domains surrounded by flexible domains that allow folding. The calcium-binding domains are homologous to epidermal growth factor and the flexible domains to proteins that bind transforming growth factor- β . Fibrillin is secreted as covalently connected, head-to-tail dimers. The N- to C-terminal regions are central to the folding that provides the beaded appearance. Folding is stabilized by transglutaminase cross-linking between glutamine and lysine residues and various small proteins that bind to the folded (beaded) fibrillin. The central region of each molecule is free of associated proteins and consists of partially folded calcium-binding domains that stretch, expanding the bead periodicity from 56 nm to 70 nm. There are three homologous fibrillin molecules (fibrillin-1 through 3). Disruption of elastic tissue scaffolding due to mutations in fibrillin-1 and -2 affect the aorta, eyes, and skin. Some fibrillin mutations also cause abnormal bone growth (Marfan's syndrome) perhaps due to an uncontrolled activation of latent TGF- β attached to microfibrils together with any bone morphogenetic protein family member except BMP-1.

interfere with a normal interaction between TGF- β and BMP on the fibrillin surface. Mutations of stromal proteins that affect the teeth are listed in Table 7.1. It should be clear that fibrillin is absent from teeth and bones, which are essentially calcified type I collagen.

6.2.1. Elastin

Animals placed on a copper-deficient diet exhibit a decreased content of elastic fibers and suffer aneurysms of the aorta. This observation suggested an impaired cross-linking of elastin, and led to the isolation of a soluble precursor, *tropoelastin* (~72,000 kDa), from the aortas of the copper-deficient animals. Elastin is encoded by a single gene with an exceptionally high intron/exon ratio. It is mainly expressed by fibroblasts and chondroblasts. Sequencing the tropoelastin polypeptide identified alternating short hydrophilic and hydrophobic domains. The hydrophilic domains are rich in lysine (K) with adjacent alanine (A) or proline (P) residues that become cross-linked (KA and KP domains). The hydrophobic domains are rich in valine (V), proline (P), and glycine (G), often as VPGVG or VGGVG repeats with or without alanine (A) and are ultimately responsible for the elasticity (Fig. 6.4a).

After tropoelastin is synthesized into the rough endoplasmic reticulum, its signal peptide is removed by a protease. The hydrophobic domains in the molecule bind to a 67-kDa chaperone, preventing self-aggregation and additional proteolysis. [*Chaperones are proteins that mediate the folding of proteins and sometimes stabilize conformations that promote binding to other proteins.*] The hydrophilic, C-terminal domain is a KP domain which does not bind the chaperone. This KP domain mediates the attachment of tropoelastin, mostly to fibrillin-2 microfibrils with bound microfibril-associated glycoprotein-1,

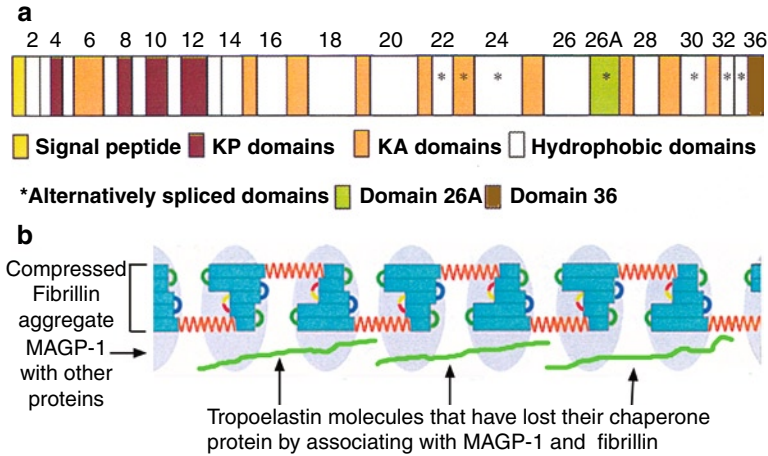


Fig. 6.4 Human tropoelastin domains. Each domain corresponds to an exon, which is irregular in size; the gene has a ratio of intron to exon DNA of about 20:1, one of the largest known. This ratio is only about 8:1 in fibrillar collagens. The signal peptide is cleaved in the endoplasmic reticulum, leaving tropoelastin. **(a)** *Elastin has hydrophilic and hydrophobic domains.* The lysine-rich KA and KP domains (colored) are hydrophilic and involved in cross-linking. They are interspersed within mostly longer hydrophobic regions (white). Asterisks indicate domains that may be included or spliced out (alternative splicing), permitting minor differences in elastin structure for different tissues. The C-terminal domain (domain 36) is essential for the interaction of tropoelastin with fibrillin to form elastic fibers (illustrated in **b**). Domain 26 is critical for the succeeding step, coacervation, whereby the fibrillin–tropoelastin aggregates form filamentous structures that then cross-link and develop into elastic fibers; recombinant tropoelastin lacking in domain 26 neither coacervates nor cross-links. Domain 26A is an exceptionally hydrophilic region that is usually spliced out. **(b)** *Association of fibrillin with elastin.* Fibrillin-associated protein MAGP-1 (light blue ovals) binds to elastin (green) by displacing the chaperone (**a** – Reprinted from Mithieux S, Weiss AS. (2005) “Elastin.” *Adv. Prot. Chem.* 70:437–461; with permission from Elsevier. **b**–Original figure derived by adding a representation of elastin to Fig. 6.2)

(MAGP-1; Fig. 6.4b). The positively charged lysine residues of tropoelastin interact with the negatively charged fibrillin, forcing release of the chaperone and exposing the tropoelastin hydrophobic domains to the aqueous environment. Exposure of one such domain two-thirds of the way to the tropoelastin C-terminus causes the microfibril-bound tropoelastin molecules to repel water by coaggregating (*coacervation*). Coacervation causes the attached microfibrils to enclose the tropoelastin molecules, limiting their aqueous exposure. Coacervation is essential for the tropoelastin molecules to align for cross-linking. Later in life, the lack of expression of fibrillin-2 makes repaired elastic fibers weaker than those laid down during development.

Cross-linking of the tropoelastin molecules within the tropoelastin–fibrillin aggregates is mediated by lysyl oxidase, the same enzyme responsible for cross-linking collagen fibers. In the KA domains, lysine residues are typically found in clusters of two or three amino acids, separated by two or three alanine residues. These regions are proposed to be α -helical with 3.6 residues per turn of helix, which has the effect of positioning two lysine

side-chains on the same side of the helix, facilitating the formation of desmosine cross-links (Fig. 6.5). In the KP domains, the lysine pairs are flanked by prolines and bulky hydrophobic amino acids. Desmosine and isodesmosines have not been found in association with KP domains, probably due to the steric constraints imposed by the prolines. The loss of many positively charged lysine residues following cross-linking makes elastin fibers among the most insoluble proteins in the body and much less soluble than tropoelastin. It is not clear exactly how lysyl oxidase accesses the KA and KP domains at the center of the tropoelastin–fibrillin aggregates.

The hydrophobic domain of elastin is a compact, dynamic structure which forms short-lived interconverting structures: distorted β strands, fluctuating β turns, and buried hydrophobic residues. Nevertheless, the numerous amide groups in the peptide bonds can still hydrogen bond with water. The overall structure is therefore a compact amorphous

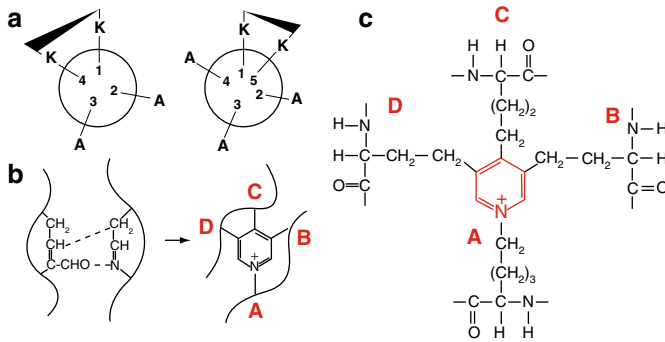
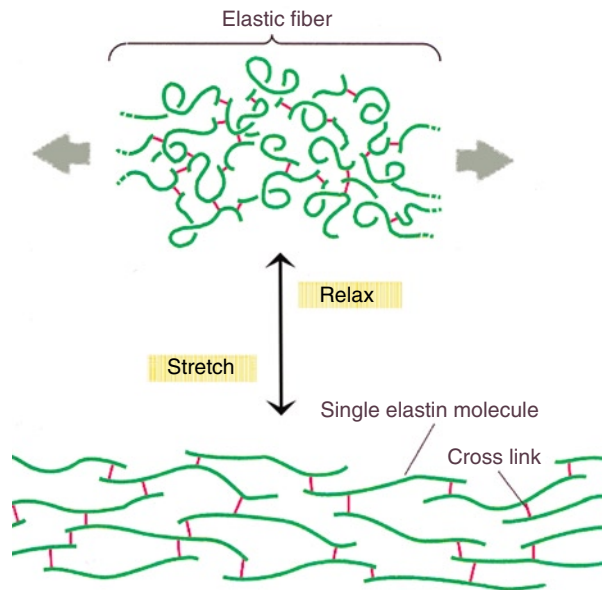


Fig. 6.5 Elastin cross-linking. **(a)** *Lysine residue relationships.* In any α -helical domain, each residue is related to the next one by a translation of 1.5Å along the helical axis and a rotation of 100°, forming a rod-like main chain structure with the side chains extended outward in a helical array as indicated in the figure. For elastin KA sequences (...Lys-Ala-Ala-Lys... and ...Lys-Ala-Ala-Ala-Lys...) the 140° rotation makes the fourth lysine side chain close to the first lysine. The lysine in position-5 is equally close, but on the opposite site. Absent from KA sequences are lysine residues in position-3 or position-6, which would lie on the opposite side of the helix from lysine 1 and be unable to participate in desmosine formation (From Rosenbloom J, et al. (1993 Oct) "Extracellular Matrix 4: The Elastic Fiber." FASEB J. 7(13):1208–1218). **(b)** Tropoelastin lysyl oxidase action. Oxidation of one of these lysine residues by lysyl oxidase results in a "within chain" cross-link precursor (dehydro-lysinorleucine, Fig. 4.8). The latter spontaneously interacts with allysine residues from a similar pair of lysine residues in an aligned, adjacent tropoelastin molecule to give desmosine. As with collagen, the cross-linking is due to spontaneous reactivity of the lysyl oxidase-generated allysine aldehyde (Adapted by permission from Macmillan Publishers Ltd. Rosenbloom J. (1984 Dec) "Elastin: relation of protein and gene structure to disease." Laboratory Investigation 51(6):605–623). **(c)** Structure of desmosine showing the lysine residue attachment sites. Isodesmosine is similar except that attachment C is at carbon 5, between A and B instead of between D and B. KP domain lysine residues also participate in cross-links, corresponding to those not involving hydroxylysine in collagen: dehydro-lysinorleucine (double) or dehydromerodesmosine (triple) cross-links. Elastin has an interchain lysine-derived cross-link at about every 68 residues: i.e., involving all but ~5 of the 34 lysine residues of tropoelastin (From Figure 11.26 in Biochemistry, L. Stryer, 3rd Ed. 1988. W.H. Freeman & Co., New York)

structure less densely packed than in soluble proteins such as albumin or hemoglobin. The elasticity is due to the increase in entropy from hydrophobic residues exposed to the aqueous environment after stretching and favors collapse when the stretching force is removed (Fig. 6.6). Elastin becomes brittle if dried and *in vivo* it remains in a *dynamic* relaxed state, not a *conformationally rigid* state like fibrillin.

Elastin is synthesized and secreted mostly during early development and it has a half-life of ~70 years. Otherwise, it is only made after an injury or infection, which induces white blood cells, especially neutrophilic granulocytes (neutrophils) to the site (Sect. 13.2.3). One of the many proteolytic products of neutrophils is elastase (Sect. 8.3.1), which hydrolyzes elastin at sites between the cross-linked region. Cleavage is most common on the C-terminal side of valine bonded to alanine (i.e., between val–ala residues).

Fig. 6.6 Elastic fiber structure. Elastin is cross-linked at the KA and KP domains. The remainder of the molecule is hydrophobic. When stretched these hydrophobic regions come into excessive contact with the water and return to a more globular structure on relaxation (Adapted from Fig. 19-52 in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002, Garland Science, Taylor & Francis Group, New York)



Elastin is encoded by a single gene, tropoelastin, which has an exceptionally high intron/exon ratio. It is expressed by fibroblasts and chondroblasts along with microfibrillar components. In the endoplasmic reticulum, tropoelastin remains soluble by binding to a chaperone. On secretion, the chaperone-free C-terminal region of tropoelastin and its many, positively charged lysine residues are attracted to negatively charged fibrillin-2 and an associated glycoprotein on co-secreted microfibrils. The bound tropoelastin now has its many hydrophobic domains exposed to water. One such hydrophobic domain causes the tropoelastin to coacervate so that the tropoelastin molecules come together at the center of the microfibrils instead of outside. The lysine residues can then react with lysine oxidase and cross-link into a large elastin aggregate within the microfibrils. Stretching exposes the many central, disorganized hydrophobic regions to water so that it collapses when the force is removed. Elastin has a dynamic relaxed state, not a conformationally rigid state like fibrillin.

6.3.1. Glycosaminoglycans

The most abundant glycans in connective tissue ground substance and cartilage are the glycosaminoglycans (GAGs): *hyaluronan*, and *protein-bound glycosaminoglycans* (proteo-GAGs) called chondroitin, keratan and heparin sulfates. Hyaluronan is a polymer of glucuronate, the salt of glucuronic acid at physiological pH, and *N*-acetyl glucosamine (Fig. 6.7). Hyaluronan is involved with other glycosaminoglycans in diverse physiological functions: matrix structure, development, and ovulation. Various glycosaminoglycans are involved in blood coagulation and pathological conditions. Table 6.1 lists the various proteo-glycosaminoglycans and their functions. Heparin and dermatan sulfate are especially important glycosaminoglycans on the luminal surface of intact endothelial cells. At this

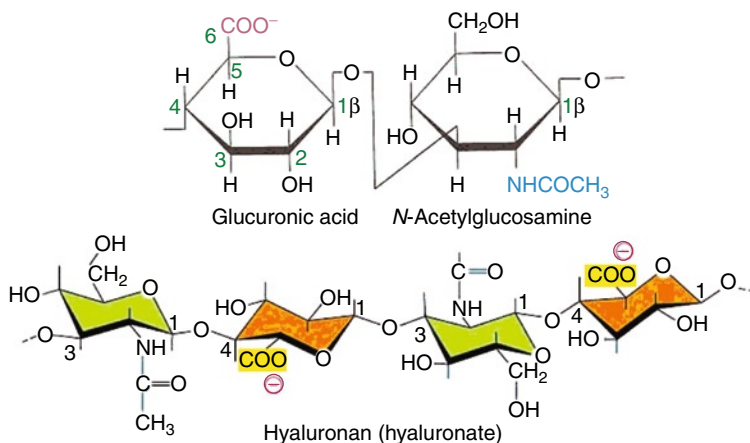


Fig. 6.7 Hyaluronan structure. Hyaluronan is composed of a repeating dimer of glucuronate attached to *N*-acetylglucosamine. *Top half* shows the ring configurations of the glycans and the *bottom half* shows the boat configurations. The carbon atoms of glucuronate (*top left*) are numbered from the anomeric OH group carbon (C_1). The OH group attached to C_1 may be configured α (*down*) or β (*up*) in the D-series sugars (determined by the configuration of H and OH groups around C_5 of a hexose, i.e., glucose and fructose), and it freely rotates between these two configurations through a straight chain aldehyde configuration in which the ring is broken. This end of the free monosaccharide and of hyaluronan is also known as the reducing end because it reduces cupric to cuprous salts (see Fig. 15.08 for a diagram of the monosaccharide ring and straight chain forms). In hyaluronan, the C_1 OH group is always connected to the adjoining residue in the β configuration as shown. Glucuronate is glucose in which the CH_2OH group attached to C_6 is oxidized to a carboxyl group (COO^-). *N*-acetylglucosamine (*top right*) is an aminoglycan, glucose with its C_2 the OH group replaced by an amine (forming glucosamine) and then one of the two hydrogen atoms of the amino group replaced by an acetyl group. In hyaluronan, each glucuronate residue is attached $\beta 1 \rightarrow 3$ to *N*-acetylglucosamine and each *N*-acetylglucosamine is connected $\beta 1 \rightarrow 4$ to the adjacent glucuronate (*bottom half*) (*Upper half* is from Fig. 18-15 in *Biochemistry*, L. Stryer, 4th Ed. 1995. W.H. Freeman & Co., New York and *lower half* is Fig. 19-38 in *The Molecular Biology of the Cell*, B. Alberts et al., 4th Ed. 2002, Garland Science, Taylor & Francis Group, New York)

Table 6.1 Some common proteo-glycosaminoglycans (proteo-GAGs)

Proteoglycan	Core protein mol wt	Type of GAG chains	No. of chains	Location	Functions
Aggrecan	210,000	Chondroitin and keratan sulfate	~130	Cartilage	Mechanical support; large aggregates with hyaluronan
Betaglycan	36,000	Chondroitin or dermatan sulfate	1	Cell surface and matrix	Binds TGF-beta
Decorin	40,000	Chondroitin or dermatan sulfate	1	All connective tissues	Binds type I collagen fibrils and TGF-beta
Perlecan	600,000	Heparan sulfate	2–15	Basal laminae	Structural and filtering function in basal lamina
Syndecan	32,000	Chondroitin and heparan sulfate	1–3	Epithelial cell surface	Cell adhesion; binds fibroblast and other growth factors

Adapted from Table.19-4 in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York

site, the inner surface of blood vessels, they inactivate blood clotting outside sites of injury (Sect. 11.5.1). In addition, heparin is stored in mast cells within the blood and may be released to inhibit excessive blood clotting after injury or infection. Heparin on the cell surface is also part of the fibroblast growth factor receptor associated with tissue development and repair (Sect. 13.2.5).

Glycosaminoglycans are solubilized from stromal or other tissues by extracting the source tissue with dilute acid or alkali. Hyaluronan is electrostatically bound to specific proteins called *hyaladherins*, which possess a structural domain of ~100 amino acids termed a *link module*. Other glycosaminoglycans are O-linked to serine and threonine residues of polypeptides and these bonds hydrolyze before the rest of the polysaccharide. The protein moiety precipitates when trichloroacetic acid or ammonium sulfate is added to the cooled mixture. The composition of the GAGs (including hyaluronan) was identified by chromatographic separation of the purified polysaccharides, followed by their hydrolysis in boiling 1.0 M HCl for 2–4 h and identification of the individual monosaccharide components.

Hyaluronan is synthesized by a transmembrane enzyme (hyaluronan synthetase) from fibroblasts, chondroblasts, and osteoblasts and is degraded by liver endothelial cells. The inner (cytosolic) face of hyaluronan synthetase binds to uridine diphosphate (UDP) activated precursors, UDP-glucuronate and UDP-*N*-acetylglucosamine (Fig. 6.8a). There are two binding domains, one for glucuronate and one for *N*-acetyl glucosamine that the synthetase alternates, so that the correct order of insertion into the polymer is maintained. The UDP attached to the monosaccharide added to the chain is not hydrolyzed until the next UDP-monosaccharide is added (Fig. 6.8b). The chain therefore grows by the incoming UDP-monosaccharide hydrolyzing the UDP attached to the hyaluronan’s *reducing end*. By

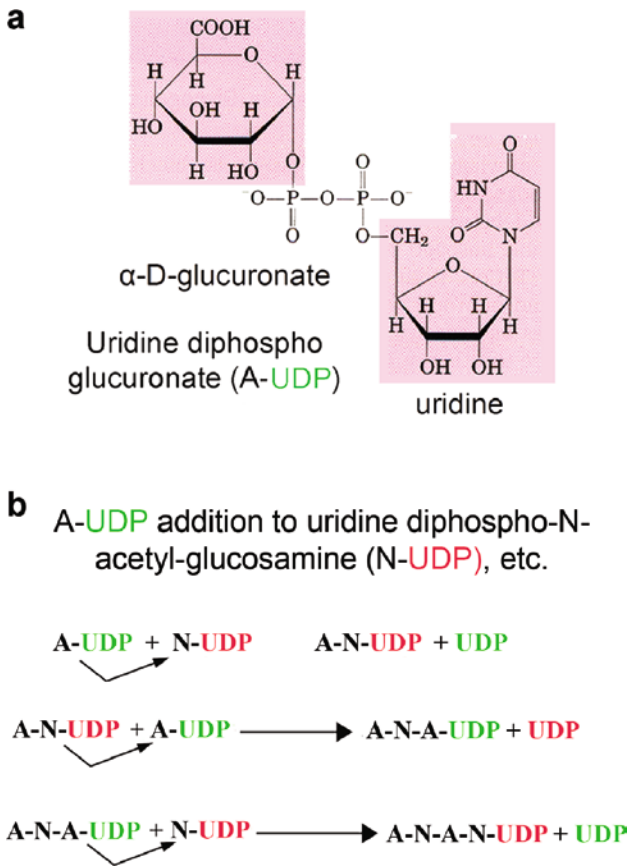


Fig. 6.8 Hyaluronan biosynthesis. Hyaluronan is synthesized by extending the reducing end where uridine diphosphate (UDP) is attached to the glycan residues. **(a)** Structure of UDP-glucuronate (A-UDP). **(b)** Synthesis of hyaluronan. *Top:* The chain begins when the UDP-glucuronate adds UDP-aminoglycan (N), which retains its UDP (red UDP terminus). *Middle:* The next glycan must be the UDP-glucuronate (A), which displaces the terminal UDP (green UDP terminus). *Bottom:* The third glycan must be another UDP-aminoglycan (N) molecule, which displaces the terminal UDP of UDP-glucuronate (A) at the end of the chain when it adds (red UDP terminus). This synthesis is the reverse of the synthesis of most other polysaccharides (e.g., glycogen, starch, and other glycosaminoglycans), which elongate by adding to the nonreducing end. The UDP remains attached to the first residue of the chain and each donor loses its UDP when it adds to the chain (**a** – Slightly modified from: Lehninger Principles of Biochemistry, Nelson, D.L. and Cox, M.M., 4th Ed. 2005. W.H. Freeman & Co., New York; **b** – Adapted from Tlapak-Simmons, V. et al. (2005 Apr 1) “Hyaluronan biosynthesis by class I streptococcal hyaluronan synthases occurs at the reducing end.” J. Biol. Chem. 280(13):13012–13018)

contrast, glycogen or starch are extended from the *non-reducing* end; the incoming NDP is hydrolyzed, not the NDP at the end of the polymer.

The length of the polymer is probably controlled by the intracellular supply of the two UDP-monosaccharides. If one monomer becomes depleted, chain lengthening stops and the terminal UDP is hydrolyzed. The reducing end of the polymer in the cytosol is now free to pass through the membrane but it may remain bound to the cell surface. Hyaluronan is degraded into large fragments in the stroma by hyaluronidase from the same cells that make hyaluronan. Hyaluronan fragments diffuse into the lymphoid circulation and eventually the blood. In the liver, endothelial cells possessing hyaluronan receptors bind to and endocytose the fragments, passing them to lysosomal vesicles where they are degraded to glucuronate and *N*-acetylglucosamine.

The numerous negative charges on the hyaluronan molecule cause an extended chain conformation, allowing hydrogen bonding to salts and water in the extracellular fluid. This interaction expands the extracellular space and provides a long, water-absorbent region to which various proteins bind, forming a gel-like ground substance. The fibrous proteins of the extracellular matrix (collagen etc.) lie within and strengthen the gel. Hyaluronan is secreted first during organ formation, or when damaged tissues are repaired, and is replaced as the tissue develops (Sect. 13.2.5). The large area that a hyaluronan molecule occupies compared with tropocollagen or albumin is illustrated in Fig. 6.9.

Proteo-GlycosAminoGlycans (Proteo-GAGs) contain chondroitin-, dermatan-, keratan-, or heparin-sulfate and have the properties of proteins called mucins (Sect. 12.3.1). The names refer to the tissues where they were first identified and are usually most prominent. Dermatan and keratan sulfate are named for the dermis of the skin and gingiva, chondroitin for cartilage and heparin for liver.

Figure 6.10 compares the composition of various proteo-glycosaminoglycans with hyaluronan. UDP-glucuronate is epimerized to UDP-iduronate in dermatan and heparin sulfate (bottom row). UDP-galactose is incorporated instead of UDP-glucuronate in keratan sulfate (top, right). UDP-*N*-acetylglucosamine is incorporated into keratan and heparin, but UDP-*N*-acetylgalactosamine is incorporated into chondroitin and dermatan. Once

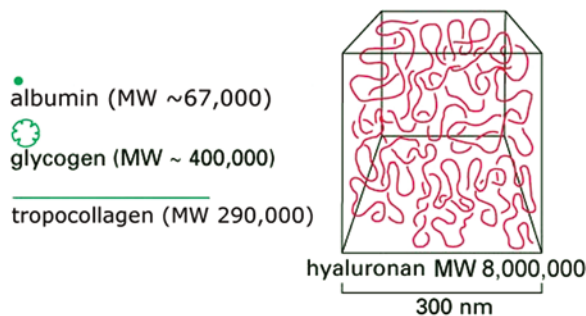


Fig. 6.9 Hyaluronan and other molecules. The volume of a single, hydrated hyaluronan molecule is compared with those of monomeric tropocollagen (triple helix), glycogen and albumin (Adapted from Fig. 19-37 in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002, Garland Science, Taylor & Francis Group, New York)

the polymer is made, the aminoglycan of chondroitin and keratan is sulfated at the C-6 position, whereas that of dermatan is sulfated at the C-4 position. Heparin sulfate is a unique dimer. The repeating link in the dimer is $\beta 1,4$ (not $\beta 1,3$ as in the other glycosaminoglycans), and each dimer is linked $\alpha 1,4$. Heparin is additionally unique because its iduronate residues are sulfated (in the 3'-OH position) and its *N*-acetyl group on the glucosamine residues is replaced with an *N*-sulfate group.

Glycosaminoglycan synthesis occurs in the Golgi, which contains enzymes that catalyze the sequential addition of uronate and aminoglycans. Serine hydroxyl groups in the

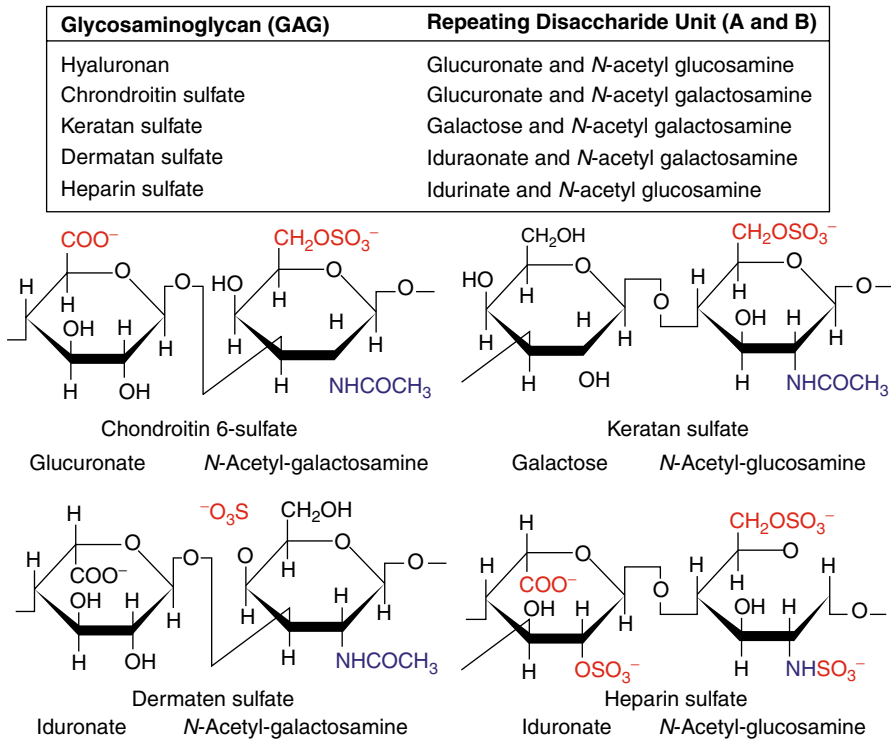


Fig. 6.10 Other glycosaminoglycan structures. Structures of chondroitin, keratan, dermatan, and heparin sulfate (The hyaluronan structure is shown in Fig. 6.7). In proteo-glycosaminoglycans containing *N*-acetylglucosamine (chondroitin and dermatan), the uronate is connected $\beta 1 \rightarrow 3$ to *N*-acetylglucosamine and $\beta 1 \rightarrow 4$ to uronate in the succeeding dimer (*left half* of figure). If the aminoglycan is a glucosamine derivative, the galactose is connected $\beta 1 \rightarrow 4$ to *N*-acetylglucosamine and $\beta 1 \rightarrow 3$ to the succeeding uronate for keratan sulfate (*top right* of figure), but iduronate is connected $\beta 1 \rightarrow 4$ and $\alpha 1 \rightarrow 4$ in heparin sulfate (*bottom right* of figure). (One way to remember the different sugars is to think of hyaluronan having only glucose derivatives, keratan as having *N*-acetyl galactosamine instead of *N*-acetyl glucosamine, chondroitin sulfate as containing glucuronate and galactosamine and dermatan as containing idurionate in which the uronic sugar points down compared with glucuronate) (Adapted from Fig. 18-15 in Biochemistry, L. Stryer, 4th Ed. 1995. W.H. Freeman & Co., New York)

core polypeptide have a surrounding domain that activates an enzyme to add UDP-xylose. Additional enzymes add other monosaccharides, forming a chondroitin sulfate linker glycan (Fig. 6.11) that may be a recognition signal for activating the appropriate synthetase to attach the first GAG monosaccharide. Keratan sulfate linkers resemble those of salivary glycoproteins (Sect. 12.2.1) and mucins (Sect. 12.3.1). A class of keratan sulfate originally found in the cornea of the eye (KS-I) is N-linked to asparagine by N-acetylglucosamine and mannose residues. The attachment is first synthesized on dolichol phosphate, transferred to the core protein and reduced in size to contain only the attachment glycans (Fig. 12.3). The other two keratan sulfate types are O-linked to serine or threonine by N-acetyl galactosamine as described for salivary mucins.

Synthetases in the Golgi add each UDP-activated monosaccharide and aminoglycan alternately to the core protein linker monosaccharide. The synthetases are homologous to hyaluronan synthetase, but their mechanism of addition is to the **non-reducing** end like glycogen or starch synthesis. Each incoming monosaccharide releases its UDP as it is added to the C3 or C4 –OH group of the growing glycosaminoglycan chain. During glycosaminoglycan synthesis, the GAGs are sulfated by enzymatic transfer of an acidic sulphate residue from 3'-phosphoadenylyl-phosphosulfate. The completed polymers are much smaller than hyaluronan, only 70–200 residues in length (14–40 kDa).

The glycosaminoglycans are beta-linked polysaccharides. Hyaluronan is unique in that it is not O-linked to a protein in the Golgi, not synthesized at the nonreducing end and not sulfated. Hyaluronan is also much longer than other glycosaminoglycans and entirely composed of glucose derivatives. Hyaluronan is synthesized by an incoming UDP-monosaccharide displacing the UDP at the reducing end of the polymer, whereas the others are synthesized by loss of their UDP residue when it is added to the non-reducing end like glycogen or starch. The protein-linked glycosaminoglycans are chondroitin sulfate, dermatan sulfate, keratan sulfate, and heparin sulfate. Except for some short chains of heparin sulfate at cell surfaces, all four sulfated glycosaminoglycans are found covalently linked to a protein.

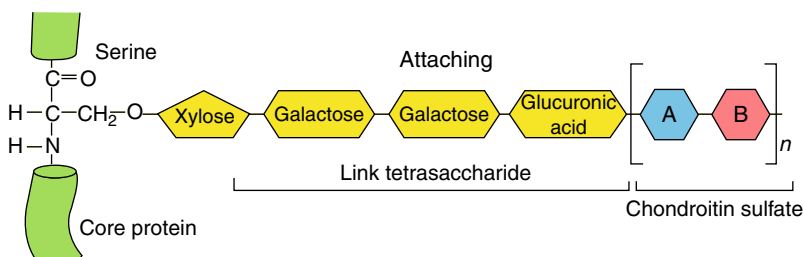


Fig. 6.11 Linker glycan for chondroitin sulfate synthesis. In the Golgi, the serine residue –OH group on a protein activates a specific synthetase in the Golgi membrane to transfer UDP-xylose. UDP is lost in making the attachment shown. UDP-activated galactose and glucuronate are then added by other synthetases before chondroitin sulfate synthetase is activated; each donor loses its UDP unlike hyaluronan synthesis. Keratan sulfate is added different linker glycans (Adapted from Fig. 19-39 in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York)

6.4.1.

Proteo-Glycosaminoglycan Core Proteins and Cartilage Collagens

Aggrecan is a large keratan sulfate/chondroitin sulfate-linked proteoglycan (2,500 kDa mass) which is prominent in cartilage, but also present in brain, aorta, and tendon. Aggrecan provides a hydrated, space-filling gel caused by more than 100 polyanionic (negatively charged) glycosaminoglycan chains attached to a polypeptide core. The mass of this proteo-GAG varies slightly with age in humans (210–250 kDa). Aggrecan provides a ground substance that holds together the fibrous component of cartilage (type II collagen) and provides it with resilience. Similar amounts are present in brain, aorta, and tendon, but little is present elsewhere in the body. *Versican* is a more widely expressed, related proteoglycan with an apparent molecular mass of about 1,000 kDa. It has many fewer attached glycosaminoglycans than aggrecan and so provides a less viscous stromal ground substance than does cartilage.

Together with *neurocan* and *brevican*, which are only found in brain, they form the aggrecan family, also called the *lectican* or *hyalectan* family. The aggrecan family of proteins possesses a multi-domain structure. Their N-terminal domain binds to hyaluronan in an interaction stabilized by pair of link protein *hyaladherins*. The central domain of aggrecan contains many serine-glycine repeats that mostly attach chondroitin sulfate, and their C-terminal domain binds to oligosaccharides in the extracellular matrix or cell surface in an interaction requiring calcium ions. The C-terminal domain is made up of three smaller domains (modules); one or two EGF-like repeats, a lectin (glycan binding)-like module, and a complement regulatory protein-like module that binds oligosaccharides in a reaction stabilized by calcium ions (Fig. 6.12). This last module is a single copy of a short repeat sequence found in receptors for complement, a group of proteins from blood plasma (Sect. 3.3.2).

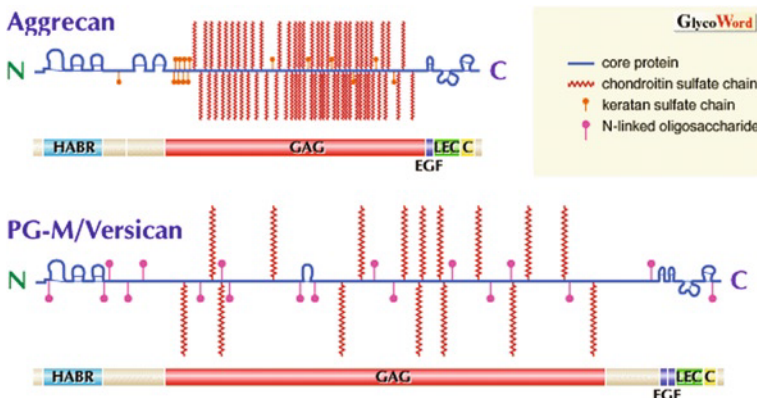


Fig. 6.12 Peptide domains and attached glycosaminoglycans in aggrecan and versican. *HABR*: Hyaluronic Acid-Binding Region; *GAG*: Glycosaminoglycan-Attachment domain; *EGF*: Epidermal Growth Factor-like repeat; *LEC*: C-type Lectin-like module; *C* (yellow): Complement regulatory protein-like module (see text) (From GlycoWord website: <http://www.glycoforum.jp/science/word/proteoglycan/PGA03E.html> (contributed by Toshikazu Yada))

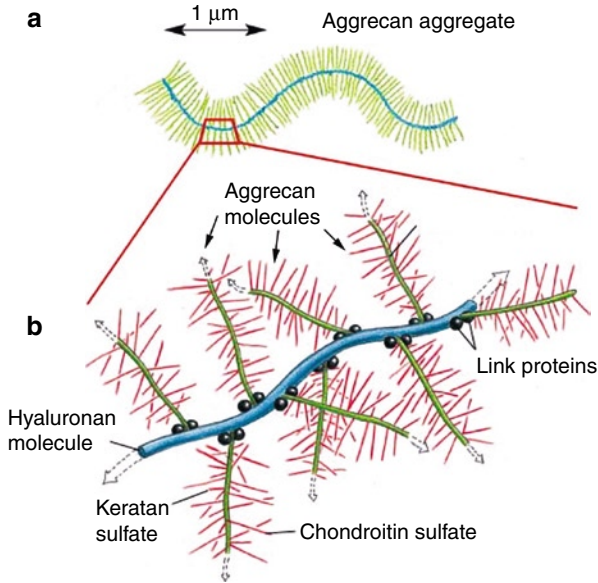


Fig. 6.13 Aggrecan and aggrecan aggregate from fetal bovine cartilage. **(a)** *Comprehensive diagram of the hyaluronan/aggrecan aggregate.* Hyaluronan = blue; aggrecan = green. **(b)** *Details of hyaluronan/aggrecan aggregate.* Link proteins attach the N-terminal (head) region of numerous aggrecan molecules to the hyaluronan polymer (blue). The aggrecan-attached chondroitin and keratan chains (red) are shown spreading out from the aggrecan polypeptide (green) (From Fig. 19-41 in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York)

Figure 6.13a shows the noncovalent aggrecan/hyaluronan complex, which comprises the ground substance (central mass) of cartilage interspersed between the type II collagen fibers. The central extended chain of hyaluronan (blue) has one end still protruding into the chondrocyte (not shown). The link proteins (hyaluronan-binding) attach the double globular head of aggrecan to the hyaluronan. The negatively charged glycosaminoglycans are partly neutralized by the sodium ions in the extracellular fluid. The glycan residues all stain strongly with uranyl acetate causing the aggrecan aggregate structure diagrammed in Fig. 6.13b to be clearly visible in an electron micrograph (not shown).

Type II collagen fibers are smaller in diameter than type I and more randomly oriented within the proteoglycan matrix. These fibers impart strength and compressibility to the cartilage matrix, so that it resists large deformations in shape as the joints absorb physiological shocks during function. Type II fibers are noncovalently cross-linked to proteoglycans by type IX collagen, a fibril-associated collagen. The triple helix of type IX collagen is composed of three separately encoded polypeptides (α_1 IX, α_2 IX, α_3 IX) that form three triple helical regions and two flexible noncollagenous domains (NC2 and NC3) in addition to the N- and C-terminal noncollagenous domains, NC1 and NC4 (Fig. 6.14a). The flexible NC3 domain of the α_2 (IX) chain has a serine residue which is covalently attached to a chondroitin or dermatan sulfate molecule and is five amino acids longer than the same

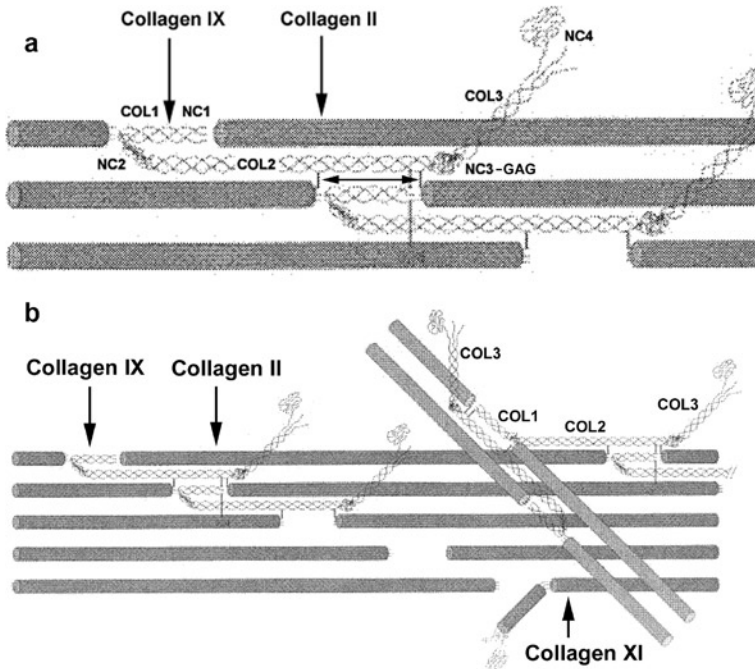


Fig. 6.14 Structures of cartilage collagens. The large gray cylinders represent the type II collagen triple helix with polypeptides running N- to C-terminal. Type II collagen is the major collagen of cartilage. Wavy lines represent the triple helix of type IX collagen, a fibril-associated collagen with interrupted triple helices (Table 3.1). (a) *Type IX collagen*: NC1 through NC4 indicate the four noncollagenous (NC) domains (see text) and COL1 through COL3 the three collagenous (COL) domains. The domains are numbered C-terminal to N-terminal. Type IX collagen fit around the triple helical region of type II collagen such that the COL3 and NC4 ends protrude. A glycosaminoglycan (GAG) is covalently attached to a serine residue at NC3 and also protrudes from the fiber. The double arrow points to two pyridinoline crosslinks at each end of the fibrillar gap regions. The right arrow cuts through a pyridinoline cross-link between the COL2 and the NC1 region of type IX collagen, and, immediately below it, also a pyridinoline between the same region and the other end of the COL 2 region. The NC1 region is also disulfide bonded. (b) *Diagram of interaction between types IX and type II collagens derived from a study of pyridinoline cross-linked polypeptides*. A collagen XI fiber with two collagenous domains separated by a central noncollagenous domain is shown at the bottom left of the figure. Both Collagen type IX and XI limit the thickness of the fibers of type II collagen. Type IX collagen also re-orientates the fibers (Adapted from Fig. 2 in Eyre DR, Wu J-J, Fernandes RJ, Pietka TA and Weis MA. Recent developments in cartilage research: matrix biology of the collagen II/IX/XI heterofibril network. *Biochemical Society Transactions*. (30): part 6 894–899, 2002: Reproduced with permission from Portland Press)

Aggrecan is a large keratan sulfate/chondroitin sulfate-linked proteoglycan (2,500 kDa mass) which is prominent in cartilage, but also present in brain, aorta, and tendon. It provides a ground substance for type II collagen, the fibrous component of cartilage, which it provides with resilience. Its N-terminal domain binds to hyaluronan an interaction stabilized by link protein. Its central domain contains many serine-glycine repeats that attach glycosaminoglycan, and its C-terminal domain binds to oligosaccharides in the extracellular matrix or attached to the cell surface. Type II collagen is associated with types IX and XI collagen which control the fiber thickness and orientation. Type IX collagen is especially important for interaction with chondroitin sulfate in the aggrecan–glycosaminoglycan aggregate. Within the glycosaminoglycan aggregate, the reducing end of the hyaluronan remains attached to the outer surface of the chondroblast and the rest of the molecule, to which the aggrecan glycosaminoglycan unit is attached, protrudes into the stroma. The fibers impart strength and compressibility to a viscous matrix enabling it to resist large deformations in shape as the joints absorb physiological shocks during function.

globular domains in the $\alpha_1(\text{IX})$ $\alpha_3(\text{IX})$ chains. The NC4 domain also protrudes, and together with the glycosaminoglycan-attached NC3 domain, anchors the cartilage collagen fibril to proteoglycans and other components of the matrix. The flexible NC2 domain of type IX collagen interacts with type XI collagen whose collagen domain has a rigid bend that limits the thickness of the type II cartilage collagen fibers (Fig. 6.14b).

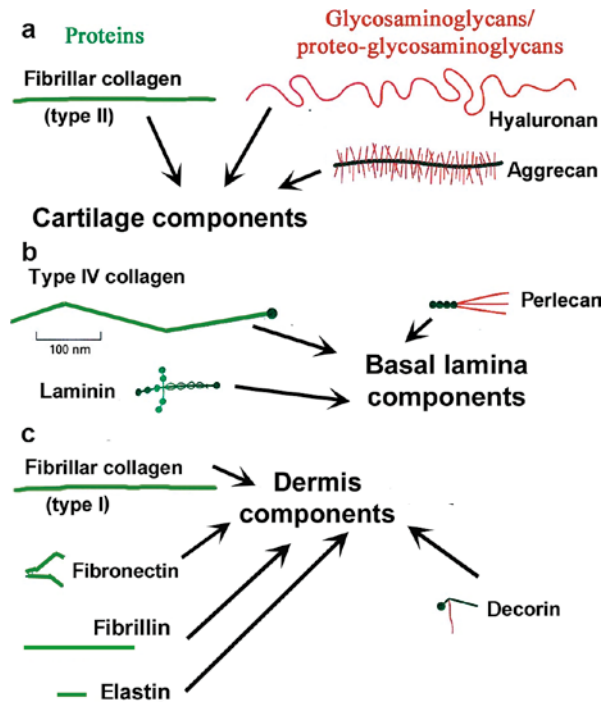
6.5.1.

Major Collagen–Glycosaminoglycan Interactions

Figure 6.15 summarizes all the major proteins and glycosaminoglycans involved in three types of connective tissues: cartilage, basal membrane, and dermis. Each of these tissues has a distinctive glycosaminoglycan and collagen. Cartilage contains type II collagen and aggrecan as discussed above. An epithelial lamina densa contains contain type IV collagen (Sect. 5.1.1) and also perlecan, a proteo-GAG containing two to 15 heparin sulfate chains. The dermis beneath the basal lamina contains type I collagen and decorin, a small molecule with a single long chondroitin or dermatan sulfate chain.

Together with type I collagen, decorin is widespread within the connective tissue stroma where it ensures proper formation and stability of collagen fibrils. It is composed of an N-terminal region to which the single chondroitin/dermatan sulfate side chain is attached, and a central region composed of ten leucine-rich repeats ($\text{LX}_2\text{LXLX}_2\text{NXL}$). The leucine repeats are surrounded by a distinct pattern of Cys residues ($\text{CX}_3\text{CXCX}_6\text{C}$) which separate the leucine repeats from the N-terminus and also make up the C-terminus of the protein. The leucine-rich region is the site of interaction with other proteins including collagen fibrils, to which decorin binds (“decorates”) with high affinity. Decorin binds to type I collagen triple helices at the major intermolecular cross-link site near the C-terminus. Biglycan

Fig. 6.15 Major extracellular matrix proteins and glycosaminoglycans. Protein is shown in *green* and glycosaminoglycans in *red*. The length of the polypeptides and glycan chains are approximately proportional to their sizes. The list is limited to major structural proteins discussed in this and the previous two chapters. (a) Cartilage. (b) Basal lamina. (c) Dermis (Modified from Fig. 19-59 in: *The Molecular Biology of the Cell*, B. Alberts et al., 4th Ed. 2002, Garland Science, Taylor & Francis Group, New York)



(not shown in Fig. 6.15) is homologous to decorin, but it contains two attached GAGs instead of only one, chondroitin sulfate in addition to dermatan sulfate. In tendons, cartilage and periodontal ligament, changes in biglycan binding to collagen or proteo-GAG aggregates activate TGF- β after tissue damage, whereas the fibrillin-mediated activation (Sect 6.1.1) may associate more with developmental changes.

Proteo-glycosaminoglycans determine the structure of cartilage, the permeability of basement membranes to small molecules, the stability of collagen fibrils, and the response of tendons, cartilage and periodontal ligaments to injury.

Collagen synthesis and degradation are central to the well-being of the teeth and periodontium. Section 1 is an overview of collagen synthesis and secretion with special emphasis on the fibrillar collagens. Section 2 describes mutations of fibrillar collagen, the associated diseases, and the effects of these and other stromal protein mutations on tooth structure. Section 3 describes the *enzymes and their cofactors that process procollagen* in the endoplasmic reticulum. The chapter concludes with a discussion of the role of ascorbate in collagen synthesis, the changes associated with its deficiency (scurvy), and its function as a reducing agent (antioxidant) in plants and animals (Sect. 4).

7.1.1. Intracellular Collagen Synthesis

Fibrillar collagen is synthesized by fibroblasts, chondroblasts, osteoblasts, odontoblasts and cementoblasts. As each collagen α -polypeptide is synthesized, an N-terminal translocation signal moves it into the ER lumen where the signal peptide is removed by a protease. The resulting product is *procollagen*, a tropocollagen extended domain with small folded domains at its N- and C-terminus (Sect. 4.2.1). The tropocollagen domains are hydroxylated at selected proline and lysine residues, glycosylated at some hydroxylysine residues, and form trimers at the C-terminal propeptide regions within the ER lumen (Fig. 7.1).

Trimer formation begins when a domain within the C-terminal $\alpha 1$ -propeptide spontaneously binds to an acceptor domain in the C-terminal $\alpha 2$ -propeptide. The heterodimer then attaches a second $\alpha 1$ C-terminal propeptide to form the trimer, 2 $\alpha 1$ chains and one $\alpha 2$ chain. The heterotrimer is stabilized by cystine cross-links catalyzed by *protein disulfide isomerase*, β -subunits of *proline hydroxylase* whose α -subunits hydroxylate the tropocollagen proline residues. Lysine is hydroxylated by *lysine hydroxylase* which is homologous to the proline hydroxylase α -subunits only (Sect. 7.3.1). After hydroxylation, glycosylation and cross-linking are completed, a soluble chaperone called hsp47 replaces proline hydroxylase and guides triple helix formation across the tropocollagen domains (Fig. 7.1b). This last process is reminiscent of the chaperone which causes newly synthesized elastin polypeptides to associate with fibrillin (Sect. 6.2.1).

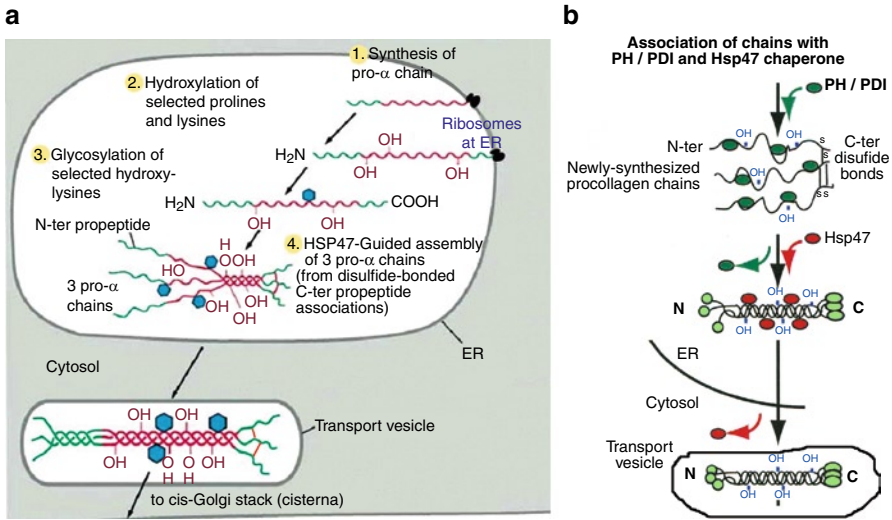
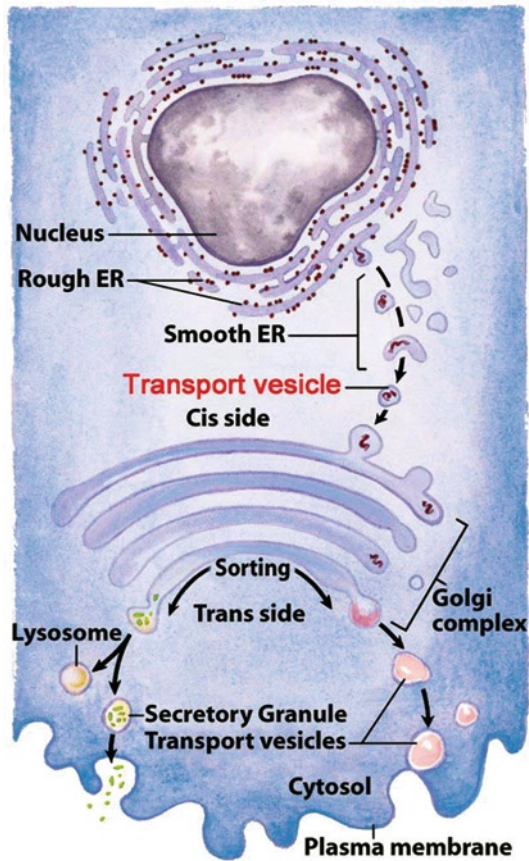


Fig. 7.1 Collagen synthesis and processing in the endoplasmic reticulum. **(a)** *Synthesis and processing of procollagen.* Steps 1 through 4 are explained in the text. The N-terminal propeptide (green on left) contains a short triple helical region that is removed with the propeptide. The C-terminal propeptide (green) is larger than the N-terminal propeptide. The tropocollagen region (red) is much larger than illustrated. Blue hexagons indicate position of the hydroxylated lysine residues with attached glycan. **(b)** *Procollagen triple helix formation.* Proline hydroxylase (PH, dark green oval) is part of the inner wall of the endoplasmic reticulum. It binds to individual polypeptides and catalyzes proline hydroxylation. Following hydroxylation, the β subunit of PH, disulfide isomerase (PDI), catalyzes the formation of triple helical tropocollagen domain by disulfide bonding between cysteine residues on the C-terminal propeptides. Hsp47 (red oval) is a chaperone that binds to the disulfide bonded tropocollagen and completes the triple helix formation. Procollagen α -polypeptides fail to form a triple helix and aggregate in the endoplasmic reticular lumen if Hsp47 is absent. Once the triple helix has formed, the procollagen is moved into a transport vesicle and transferred to the Golgi. The N- and C-terminal propeptide regions are indicated as green circles and green ovals. **(a)** Revised and partially updated from Fig. 19–47 in *The Molecular Biology of the Cell*, Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York. **(b)** Slightly modified from Fig. 6 in Ishida, Y., et al., *Molecular Biology of the Cell*, Vol. 17, 2347–2355, May 2006; Reprinted with permission from the American Society for Cell Biology; PDF access <http://www.molbiolcell.org/cgi/reprint/17/5/2346>

The procollagen trimers are taken into transport vesicles which pass them to cis-Golgi cisternae (Fig. 7.2). There, they: (a) aggregate into dense material (procollagen bundles seen in electron micrographs); (b) lose their N-terminal propeptide, which re-enters the cytosol and inhibits the translation of additional collagen; and (c) are sorted into secretory vesicles in the trans-Golgi. Secretion strongly activates propeptide removal extracellularly (Sect. 8.2.1), generating spontaneous tropocollagen fiber formation and cross-linking (Sect. 4.2.2.). The synthesis and processing of non-fibrillar collagens are similar, except that their N- and C-terminal propeptides remain attached for use in the polymeric assemblages (Sect. 5.1.2).

Fig.7.2 Intracellular organelle path taken by collagen polypeptides before secretion. Diagram shows the rough and smooth endoplasmic reticulum, the budding of primary transport vesicles from the smooth endoplasmic reticulum to the cis-Golgi, the Golgi cisternae, and the budding of secretory or secondary transport granules (vesicles) from the trans-Golgi for secretion, delivery to lysosomes, or insertion into cell membranes from the trans-Golgi to form secretory granules or lysosomes (Slightly adapted from Fig. 27-35 in Lehninger Principles of Biochemistry. D.L. Nelson & M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., New York)



Collagen is synthesized in the endoplasmic reticular lumen. Each α -polypeptide possesses a signal sequence that guides the N-terminus into the ER where it is removed, leaving a long extended tropocollagen domain with small, folded N- and C-terminal propeptide extensions (procollagen). Proline hydroxylase has two α subunits and two β subunits. The latter is called protein disulfide isomerase and it disulfide bonds C-terminal propeptides that have self-associated into trimers of two $\alpha 1$ -chains and one $\alpha 2$ -chain. Lysine is hydroxylated by an enzyme loosely homologous to the proline hydroxylase α -subunits. After selected proline and lysine residues are hydroxylated and hydroxylysine glycosylated, chaperone hsp47 replaces proline hydroxylase and guides triple helix formation of the tropocollagen domain. Procollagen then passes through the Golgi to secretory vesicles where it forms large procollagen bundles. The N-terminal propeptides are hydrolyzed and re-enter the cytosol where they inhibit collagen α -chain synthesis. Synthesis and processing of non-fibrillar collagens is similar, except that their N- and C-terminal propeptides are not removed.

7.2.1.

Effects of Collagen Polypeptide Mutations

The fibrous collagens of all vertebrates and invertebrates possess about 340 gly-X-Y sequences encoded as groups of approximately 18 amino acids in their tropocollagen domain. The 41 to 42 exons are all multiples of 9 bases. Most are 54 bases in size, but some are also multiples of 54 bases, or combinations of 45- and 54-bases. The sequences of three such exons (Fig. 7.3), which suggest that the α -procollagen polypeptide evolved from a precursor of the N- and C-terminal domains by duplication of a 54 base exon that originally encoded 18 amino acids in the central portion of an ancient protein precursor of the procollagen N- and C-terminal domains. At about the same time, a small insertion within the C-terminal region enabled different α -polypeptide mixtures to associate. The resultant “molecular incestuous” mixing resulted in allowed the formation of collagen heterotrimers compatible with bone formation and the appearance of vertebrates from invertebrates. Invertebrate collagens are homotrimers and cannot calcify.

Mutations that interfere with collagen fiber formation mostly cause lethal or nonlethal osteogenesis imperfecta, also known as brittle bone disease. The bones break easily and apparently spontaneously. The disorder occurs in about one in 50,000 live births in the US. Osteogenesis imperfecta is clinically divided by whether the teeth are also affected. They may appear opalescent blue-gray or yellow-brown because of abnormal dentin calcification.

```

13
-Gly-Pro-Met-Gly-Pro-Ser-Gly-Pro-Arg-
22
-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp-
----- Intron -----
31
-Gly-Pro-Gln-Gly-Phe-Gln-Gly-Pro-Hyp-
40
-Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Ala-Ser-
----- Intron -----
49
-Gly-Pro-Met-Gly-Pro-Arg-Gly-Pro-Hyp-
58
-Gly-Pro-Hyp-Gly-Lys-Asn-Gly-Asp-Asp-

```

Fig. 7.3 Translated sequence of a tropocollagen exon. The 18 amino acid residues (3x6 gly-X-Y sequence) encoded as a 54 bp exon. Sequence homologies between amino acids 1–18 in exon 1 of the tropocollagen domain of the *COL1A1* gene compared with the sequence of 18 amino acid residues in exons 2 and 3 respectively. Numbering is according to den Dunnen JT and Antonarakis SE (2000). Human mutation, 15:7–12. *Underlines* show identity between exon 1 and exon 2 sequences. *Highlights* show identity between exon 1 and exon 3 sequences. The collagen genes are listed in Table 3.1. The *COL1A1* gene encodes the type I $\alpha 1$ procollagen polypeptide and the *COL1A2* gene encodes the type I $\alpha 2$ procollagen polypeptide.

Table 7.1 Stromal protein mutations affecting teeth

Mutation ^a	Disease	Symptom
<i>Type I collagen</i> mutations associated with osteogenesis imperfecta	Dentinogenesis imperfecta type I (see also Chap. 9)	Opalescent blue-gray or yellow-brown teeth because of abnormal dentin calcification
<i>Kindlin-1</i> (Chap. 5)	Kindler syndrome	
Unknown genes on chromosome 12	Ehlers–Danlos syndrome – type VIII	Aggressive periodontal disease
Laminin-5 (Chap. 5)	Junctional epidermolysis bullosa	Generalized enamel hypoplasia; increased caries
Fibrillin-1 and -2 (Chap. 6)	Marfan syndrome	Crowded incompletely developed (hypoplastic) teeth and deformities of the roots
Dentin sialophosphoprotein (DSPP), a glycoprotein of the connective tissue stroma (Chap. 9)	Dentinogenesis imperfecta type II	Changes resemble type I dentinogenesis imperfecta (Chap. 9)
Mutations of amelogenin formation and processing (Chap. 9)	Amelogenesis imperfecta	Brittle or absent enamel

^aItalic type indicates the various collagen polypeptides and an enzyme mutation described in this chapter

Occasionally, some teeth may be missing. Table 7.1 is a list of stromal protein diseases that affect tooth development.

There are two major types of spontaneous genetic mutations: a change in a nucleotide base in the organism’s DNA (point mutation), or a base deletion. Either mutation can sometimes alter intron/exon splicing boundaries, causing a more extensive mutation and serious or fatal disease. Osteogenesis imperfecta is usually caused by a point mutation of the codon for glycine, which is always encoded as GGT within the *COL1A1* and *COL1A2* genes (Fig. 7.3). Since the triple helix develops from C- to N-terminus (Fig. 7.2), *a mutation near the C-terminus interrupts helix formation more completely than the same mutation near the N-terminus and is therefore more likely to be lethal.*

7.2.2.
Ehlers-Danlos syndrome (EDS)

Other mutations of fibrillar collagen, or mutations that affect collagen-processing, cause *Ehlers-Danlos syndrome* (EDS), a group of heritable connective tissue disorders causing: skin hyperextensibility, articular hypermobility, and tissue fragility. The 3 major types are classic (EDS-I and EDS-II), hypermobility (EDS-III) and vascular (EDS-IV).

Classical EDS is usually due to type V collagen mutations that unduly limit fiber thickness. Mice made haploid for type V collagen express only half the amount in normal mice and exhibit classical EDS symptoms. Occasionally, a non-glycine residue is mutated to cysteine in type I collagen and the resulting disulfide cross-linked polypeptides also limit fiber thickness. The corresponding mutation is more common in type II collagen, where instead of classical EDS, it causes a chondrodysplasia such as dwarfism. The genetic mutations involved in joint hypermobility (EDS-III) are mostly unknown.

Vascular EDS, (EDS-IV; fragile blood vessels) is exclusively associated with mutations of COL3A1, the gene encoding type III collagen (Table 3.1). Most mutations (66%) are substitutions of glycine for another amino acid in one of the gly-X-Y triplets and nearly all the rest are the result of exon skipping.

EDS-V is an X-linked form of EDS-I in which skin fragility is prominent. EDS VI is a severe form of EDS-I associated with corneal degeneration. The genetic mutations of both are unknown, but EDS-VI has reduced lysine hydroxylase activity despite no detectable mutation of any of its three genes (Sect. 7.3.1) or of the genes involved in ascorbate metabolism (Sect. 7.4.1).

EDS VII is a combination of classical and vascular EDS caused mostly by in-frame deletions of 18 or 24 amino acid residues encoding exon-6 of COL1A1 or COL1A2, the downstream splice site for procollagen N-peptidase. Mutations of procollagen N-peptidase (Sect. 8.2.1, Fig. 8.5) also cause this form of EDS.

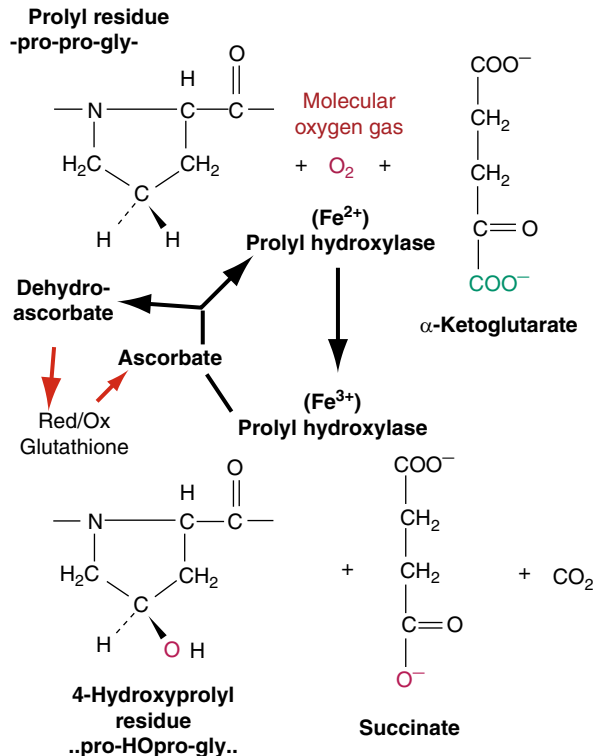
EDS VIII is associated with aggressive periodontal disease. It may be caused by a mutation in one or more genes other than the collagen gene (COL2A1) on chromosome 12. Individuals resemble Marfan's syndrome (Table 6.1), but have normal teeth. They display excessive bleeding around the knees and fragile skin, hallmarks of vascular EDS.

The similarity of collagen structure in all species suggests a common origin in which the central segment of a precursor globular protein resembling the N- and C-propeptides was repeatedly duplicated. Vertebrates contain collagen heterotrimers and bone, but invertebrates contain a simpler procollagen C-terminal domain and homotrimers which cannot calcify. Mutations of glycine residues at one of the ~340 gly-X-Y sequences in a collagen gene inhibits adequate triple helix formation and results in fragile bones (osteogenesis imperfecta), sometimes accompanied by opalescent or completely missing teeth (dentinogenesis imperfecta). Ehlers-Danlos syndrome (EDS), skin hyperextensibility, articular hypermobility, and tissue fragility, is caused by mutations that affect collagen fiber assembly. The three major types of EDS are classical, hypermobility, and vascular. Classical EDS is mostly due to mutations of type V or type I collagen that limit fiber thickness. Vascular EDS is exclusively due to mutations of type III collagen that promote vascular fragility. An unusual form of EDS, in which a gene other than the collagen gene encoded on chromosome 12 is mutated, is associated with aggressive periodontal disease.

Proline hydroxylase (PH) and *lysine hydroxylase* (LH) bind to substrate motifs on procollagen in the endoplasmic reticulum. The proline substrate motif has the sequence X-Y-Gly where X is any amino acid and Y is the proline residue to be hydroxylated. The lysine substrate motif has an analogous sequence in which Y is a lysine residue. The amino acid sequences surrounding the tripeptide substrate motif for lysine are more variable than those surrounding the proline motif. This variability facilitates or inhibits lysine hydroxylase binding and catalysis, explaining variations in the hydroxylysine content of different collagen types. The two motifs are not unique to collagen; other proteins contain these motifs and are hydroxylated similarly.

PH and LH are *mixed function oxidases*. Molecular oxygen and α -ketoglutarate are co-substrates along with procollagen. One atom of molecular oxygen is reduced by α -ketoglutarate and the other by an electron from a ferrous ion within the hydroxylase. The oxidized products are a coordinated ferric ion hydroxide at the catalytic center of the enzyme, plus succinate and carbon dioxide from the oxidation of α -ketoglutarate. The ferric ion is reduced to ferrous ion by a cofactor (ascorbate) releasing the hydroxide as a nucleophile to hydroxylate a proline or lysine residue in procollagen (Fig. 7.4). Ascorbate is oxidized to dehydroascorbate and regenerated by glutathione (Sect 7.4.1). Some of the electrons that restore ascorbate come from procollagen C-terminal cysteine residues oxidized by PDI to stabilize the procollagen trimers (Sect. 7.1.1), but the numerous proline residues to be hydroxylated require many additional electrons which are provided by glutathione (Sect. 7.4.1). PDI is also found as a single dimer in the ER where it catalyzes an exchange of disulfide bonds in many proteins.

Fig.7.4 Catalytic action of proline hydroxylase. One atom of oxygen gas (red) oxidizes ketoglutarate and appears in succinate along with CO_2 . The other oxygen atom forms a $\text{Fe}^{3+}\text{-OH}$ complex attached to the enzyme. When Fe^{3+} is reduced by ascorbate, H^+ dissociates and the O^{-1} species (red) is released to a proline residue in the polypeptide, forming a hydroxyproline residue. The enzymes recognize prolyl- or lysyl residue sequence motifs described in the text (From Fig. 11.3 in Biochemistry, L. Stryer, 3rd Ed. 1988. W.H. Freeman & Co., New York)



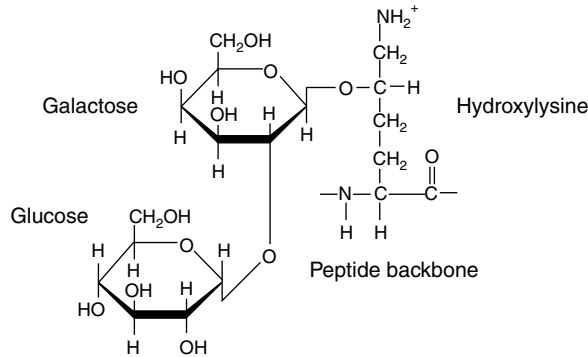


Fig. 7.5 Structure of the diglycan attached to hydroxylysine. The LH3 isoform of lysine hydroxylase attaches UDP- galactose to hydroxylysine in a β -linkage, and then α -links UDP glucose to the C_2 atom of the galactose. This reaction occurs in the endoplasmic reticulum and in the connective tissue matrix (From Fig. 11.4 in Biochemistry, L. Stryer, 3rd Ed. 1988. W.H. Freeman & Co., New York)

There are also three encoded lysine hydroxylases in the genome. LH1 and LH2 are more closely related (homologous) to each other and the PH α -subunits than LH3. All three LH isoforms are homodimers that adhere to the ER luminal membrane by a hydrophobic fold in their iron-binding domain and are unattached to PDI. Some LH3 detaches from the inner wall of the endoplasmic reticulum along with individual α -procollagen polypeptides in which all available proline and lysine residues have been hydroxylated. Once detached, LH3 has a second, unrelated enzymatic activity. It adds galactose and then glucose to certain hydroxylysine residues in each procollagen polypeptide (Fig. 7.5). The sugars are activated in the cytosol by UDP-sugar transferase and transported into the endoplasmic reticular lumen. Once glycosylated, the processed tropocollagen domain binds to chaperone hsp47 to guide triple helix formation (Sect. 7.1.1). The fate of LH3 is uncertain but it likely reattaches to the ER lumen wall.

Summary: Proline and lysine hydroxylase (PH and LH) molecules are homodimers containing an iron atom. They are attached to the luminal wall of the endoplasmic reticulum. PH (α -subunit) is held loosely by protein disulfide isomerase (PDI, β -subunit), whereas LH is directly attached. Hydroxylation is by a mixed function oxidase reaction. One atom of an oxygen molecule oxidizes the ferrous ion to a ferric ion hydroxide and the other oxidizes α -ketoglutarate, producing succinate and carbon dioxide. When ferric hydroxide is reduced by a cofactor, ascorbate, the second oxygen atom is released and hydroxylates the substrate. Ascorbate is reduced to dehydroascorbate and reoxidized by glutathione which receives electrons from various sources including PDI which oxidizes cysteine to stabilize procollagen trimers. Three homologous PH polypeptides are encoded by different genes and expressed in different tissues. There are also 3 homologous LH polypeptides, of which one, the LH3 polypeptide, glycosylates some of the hydroxylysine residues with galactose and glucose.

7.3.1. Ascorbate and Antioxidants

Ascorbate (ascorbic acid or vitamin C) is an antioxidant (reducing agent). Besides its importance as a cofactor for proline and lysine hydroxylases in vertebrates, it ascorbate protects macromolecules from oxidative damage by neutralizing *reactive oxygen species* (ROS), by-products of respiration. Cells possess large amounts of catalase, peroxidase and superoxide dismutase enzymes that rapidly neutralize these harmful agents within the cytosol (Sect. 16.3.2.). The anti-oxidant property of ascorbate is more important extracellularly where it neutralizes the ROS from leukocytes during inflammation (Sect. 13.3.1).

Dehydroascorbate forms directly when two electrons are removed from ascorbate (Fig. 7.6a, lower left), for example, after reacting with proline or lysine hydroxylase (Fig. 7.4a). Dehydroascorbate forms indirectly if ascorbate loses a single electron (for example, to an electron-deficient radical group) that first produces mono-dehydroascorbate (Fig. 7.6a, top). The two parts of the indirect reaction occur spontaneously (without an enzyme) in aqueous phase. Both ascorbate and dehydroascorbate are transported in and out of cells. Ascorbate enters through sodium-dependent transporters, and dehydroascorbate through *glucose transporters-1 and -3* (GLUT1 and GLUT3). Dehydroascorbate is immediately reduced to ascorbate inside cells, but it predominates extracellularly where it spontaneously hydrolyzes to 2,3-diketogulonate (Fig. 7.6b, lower right) with a half-life of 2–15 min.

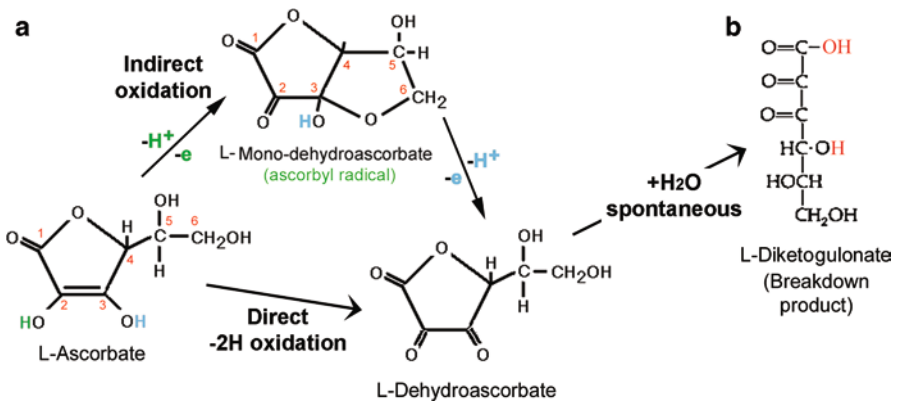


Fig. 7.6 Structures of ascorbate, ascorbyl radical and dehydroascorbate. **(a) Ring structures.** Red numbers indicate the conventional carbon atom numbering. The COO⁻ group of ascorbate (esterified to C₄) is C₁. The green and blue hydrogen atoms indicate sites of electron loss leading to mono-dehydroascorbate (ascorbyl radical) and then to dehydroascorbate (upper half of Fig. 7.6a). The mono-dehydroascorbate is stabilized by having its free electron shared among the attached oxygen atoms. The loss of a second electron and proton (blue), or of both protons and electrons directly as in the proline or lysine hydroxylase reaction (bottom half), gives rise to dehydroascorbate (Original figure). **(b) Straight chain structures.** Ascorbate is in the L-form because of the orientation around C5. Mono-dehydroascorbate is not shown. Instead, the degradation form of dehydroascorbate, L-diketogulonate is diagrammed at the *far right* (Adapted from drawings on p. 1359, Chap. 50 in Principles of Biochemistry, White, A., et al. 6th Ed. 1978. McGraw Hill Inc., New York)

Diketogulonate cannot be reduced back to ascorbate. Most animals synthesize ascorbate using the path shown in Fig. 7.7, but *primates including humans have accumulated mutations in the gene for L-gulonolactone oxidase and cannot synthesize ascorbate de novo*. They require a dietary source of vitamin C to compensate for the gradual loss of dehydroascorbate. The current Recommended Dietary Allowance (RDA) from the US Food and Drug Administration is 60–95 mg/day.

Depletion of ascorbate causes scurvy, which was first identified in sailors during long voyages between the fifteenth and seventeenth centuries. It was a common cause of illness and death if fresh fruits and meat were not eaten within 6 weeks. Citrus fruits, especially lemon and lime in which the reduced form of ascorbate stores well, were found to prevent or cure the disease. An early symptom of ascorbate deficiency is the loss of gingival and periodontal membrane fibers accompanied by loosening of the teeth. The reason is that anchoring fibrils and fibers of the gingival cuff and upper periodontium (Chap. 3) turn over every 24 h due to tooth movements that stimulate fibroblasts to replace collagen and renew the attachment continuously. Movement of blood causes a similar instability of collagen in blood vessel walls. Without

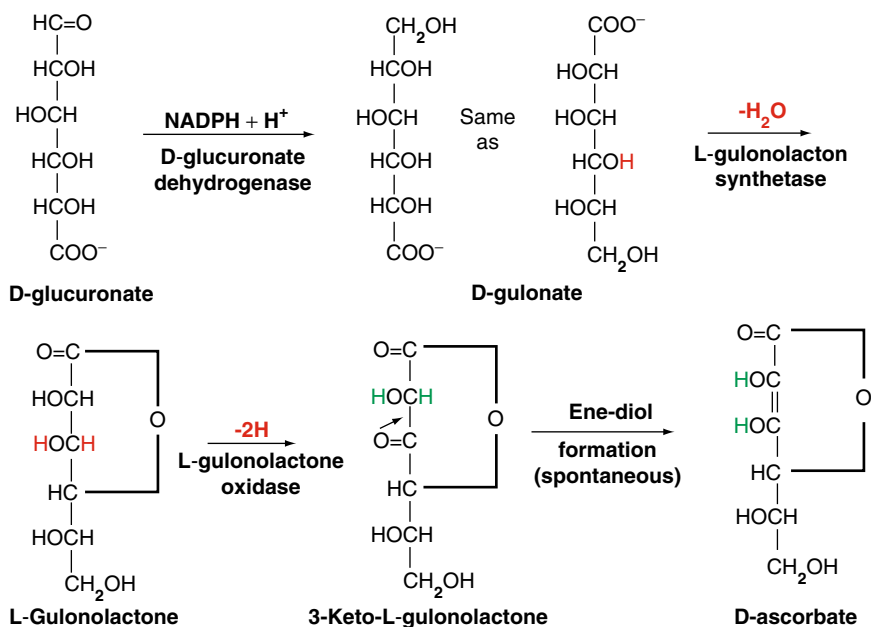


Fig. 7.7 Synthesis of ascorbate. UDP-D-glucuronate is the precursor in mammals. The precursor of endogenous ascorbate in animals is D-glucuronate, which is derived from D-glucose by glucose 6-phosphate dehydrogenase. The straight chain forms of D-glucuronate and L-gulonate, the next intermediate, are shown along with the enzymes that catalyze the changes (top row). The L-gulonate intermediate is acted on by a synthetase to lose water, forming L-gulonolactone which is oxidized to form 3-keto-L-gulonolactone. The latter spontaneously isomerizes to L-ascorbate. L-gulonolactone oxidase is inactive in humans and most other primates because of a mutation not present in other animals. Plants make large amounts of ascorbate by a different path. (Adapted from drawings on p. 1359, Chap. 50 in *Principles of Biochemistry*, White, A., et al. 6th Ed. 1978. McGraw Hill Inc., New York)

ascorbate, fibers are removed but not replaced; fresh collagen cannot be secreted. The collagen in bone turns over more slowly, but if scurvy becomes advanced, cessation of this turnover causes deep, intense bone pain. Late in the disease, a lack of type IV and other collagens surrounding blood vessels cause their degeneration, leading to potentially fatal aneurisms.

In addition to collagen metabolism and scavenging ROS species to limit inflammation as noted above, ascorbate is required for the synthesis of norepinephrine from tyrosine, of carnitine from γ -butyrobetaine whose immediate precursor is made by trimethylating lysine, for folinic acid production from folic acid. In the absence of ascorbate, the reduced activity of these processes slows nerve, energy and cardiac output, causing the affected person to become exhausted and irritable. Scurvy is the old English word for ill-tempered.

A minority of scientists, most notably Linus Pauling, believed that the failure of humans and a few other animals to synthesize vitamin C is a genetic defect that should be overcome by supplementing the diet with large amounts of vitamin C (megadoses of vitamin C). Viruses and bacteria stimulate white blood cells to release large amounts of hydrogen peroxide and oxygen-free radicals, an oxidative burst that persists after the causative agents are gone. Megadoses of vitamin C were proposed to prevent chronic damage by reducing the extent of this burst if an infection occurs. Current studies suggest that regularly taking larger doses of vitamin C (0.5–3.0 g up to 18 g/day) may inhibit some cancer cells and viruses from growing *in vitro*. There is less evidence that it prevents cancers or viral infections *in vivo*. Some better-established uses of ascorbate are as an antioxidant in foods, an antiwrinkle agent for skin, and as an antidote to ascorbate depletion associated with nickel or lead poisoning.

Ascorbate is the major antioxidant in chloroplasts and large amounts are synthesized from glucose in the cytosol of leaves by the path shown in Fig. 7.7. However, in animals glutathione (GSH) is the major antioxidant, not ascorbate (Fig. 7.8). Glutathione (GSH) is a tripeptide (Glu-Cys-Gly), in which the amino group of cysteine is attached to the γ -carboxyl group of glutamate. It is synthesized and degraded by specific enzymes in the cytosol. Two reduced glutathione molecules lose two electrons and form a disulfide bond (GS–SG). In primates, GSH transfers its electrons to dehydroascorbate or peroxidase and GSSG accumulates (Fig. 7.8, red arrows). The peroxidase action is described in connection with its inhibition by fluoride (Sect. 16.3.2; Fig. 16.8b). In collagen synthesizing cells, protein disulfide isomerase or GSH-dehydroascorbate reductase can transfer their electrons to dehydroascorbate (Fig. 7.8, green or red arrows on right). GSSG reductase obtains its electrons from NADPH from the pentose phosphate path (Fig. 7.8, blue arrow). Note that the use of protein disulfide isomerase (PDI) to oxidize cysteine residues (Sect. 7.3.1) appears limited to its association with proline hydroxylase. As its name implies, PDI primarily exchanges disulfide bonds to stabilize protein structures.

Ascorbate is a cofactor for proline and lysine hydroxylation. The oxidized form, dehydroascorbate, predominates extracellularly but degrades spontaneously. Most vertebrates synthesize ascorbate *de novo* from glucose, but the last enzyme of the path, L-gulonolactone oxidase, is inactive in primates and is therefore nutritionally essential. Ascorbate (vitamin C) is present in fresh citrus fruits and meat, but it is gradually lost because its oxidation product, dehydroascorbate, is unstable. Scurvy develops in the absence of vitamin C, preventing collagen re-synthesis in response to stress in tissues such as the gingiva, blood vessels and bone. Ascorbate also functions as a nonenzymatic scavenger of water-soluble oxidizing agents. It reduces peroxides and oxygen free radicals and is regenerated by

reductases, the protein disulfide isomerase subunit of proline hydroxylase or glutathione. Oxidized glutathione is reduced by electrons which are transferred from NADPH by glutathione reductase. The NADP⁺ is in turn reduced by the oxidation of glucose in the pentose phosphate path. Glutathione is a cofactor for peroxidases that maintain normal cell function by reducing peroxides made by reactive oxygen species on membranes and the cytosol.

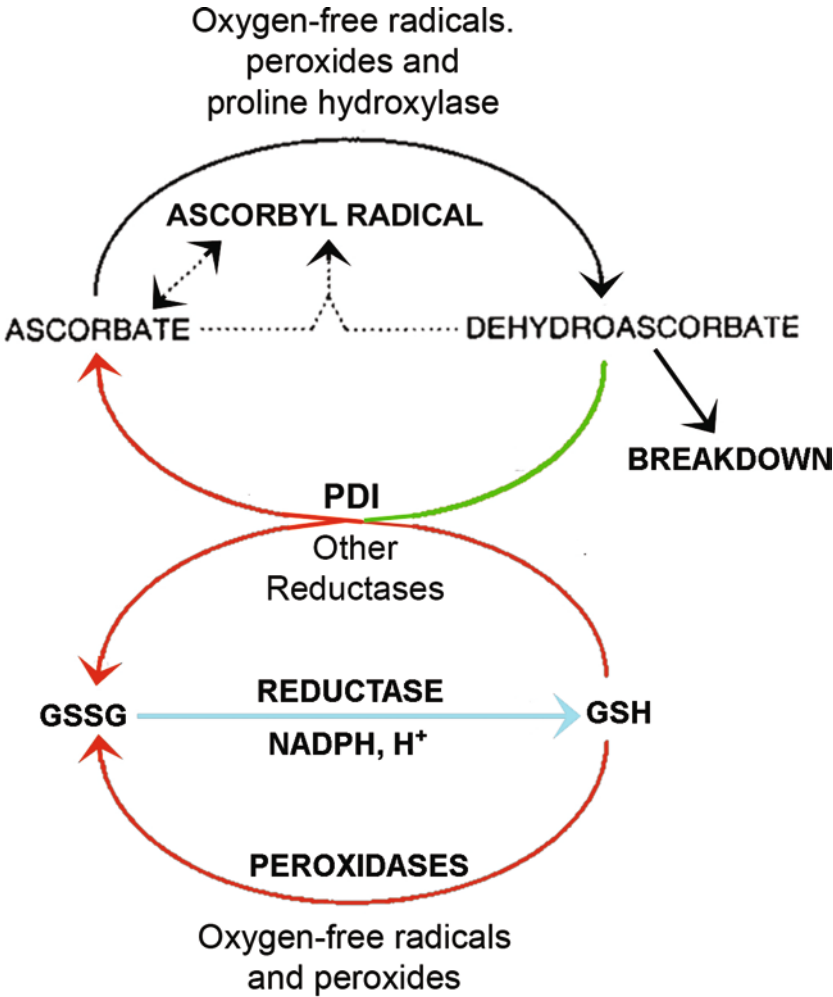


Fig. 7.8 Top half of the figure depicts the spontaneous oxidation of ascorbate by oxygen-free radicals, peroxides and proline hydroxylase (black arrow) and the reduction of dehydroascorbate to ascorbate by dehydroascorbate via PDI, or providing reducing equivalents as a cofactor for peroxidases and other reductases (upper and lower red arrows). The oxidized form (GSSG) is reduced by NADPH (straight blue arrow). (Slightly modified from Meister A., "Glutathione-ascorbic acid antioxidant system in animals." J. Biol. Chem., 269(13):9397-9400, 1994)

This chapter discusses the zinc-containing metalloendoproteinases. These enzymes remodel the stroma during development and around tissues that have been injured or stressed. Section 1 describes the three major classes of metalloproteases, their metal ion cofactors, their functions in biology, and the metzincin catalytic mechanism in procollagen and stromal protein processing. Section 2 describes the astacin and adamalysin metzincin subclasses and how they process procollagen to tropocollagen. Section 3 describes the matrilysin metzincin subclass and their roles in collagen and stromal tissue degradation and in enamel synthesis.

8.1.1.

The Zincin Enzyme Family

The zincin proteases remodel the stroma during development and around tissues that have been injured or stressed (Sects. 13.2.4). All proteases possess one of the three types of activities: (a) (Sects. 13.2.5) that cut within a polypeptide; (b) (Sects. 13.3.1) that cut at the C- or N-terminus; and (c) peptidases that cut small polypeptides. The five structural classes of proteases are listed in Table 8.1 and the enzymatic activities of the various metalloprotease classes and subclasses are listed in Table 8.2.

Most metalloproteinases contain a catalytic zinc ion bound to two histidines within a conserved motif, usually HEXXH in the one letter amino acid code where X stands for any amino acid (Fig. 8.1). These enzymes are known as *zincins*, and they comprise by far the largest clan of metalloendoprotease families. The zincin clan assignments depend on the nature of a third zinc-binding residue: glutamate (E) in *gluzincins*, aspartate (D) in *aspzincins*, and histidine or aspartate (H/D) in *metzincins*. Aspzincins are absent from the human genome (Fig. 8.1) and gluzincins encode proteases other than endoproteases (Table 8.2).

All human metalloendoproteinases are metzincins, named for a downstream methionine residue involved in regulating catalysis by mediating a critical turn that brings an adjacent tyrosine or proline residue close to the catalytic zinc ion. *Matrilysins* (also called matrix metalloendoproteinases, MMPs) are the major class of metzincin endopeptidases involved in collagen and stromal degradation. The other two classes, *adamalysins* and

Table 8.1 All the protease families

Catalytic site ^a	Genes ^b	Representative ^c	Catalytic mechanism
Serine	175	Trypsin/chymotrypsin/caspases ^d	Residue forms ^-OH from bound H_2O
Threonine	28	Proteasome enzymes ^e	Residue forms ^-OH from bound H_2O
Aspartic acid	21	Pepsin/HIV retropepsin	Aspartyl residue
Cysteine	150	Cathepsins	Cysteinyl residue
Metal ion (Zn^{2+})	187	Collagenase	Zn^{2+} ion-bound water molecule ^f

^aProtease structure is classified by its catalytic action. A subclass of the serine proteases requires, in addition, one or two histidine residues for catalytic activity

^bNumber of protease genes in the human genome

^cRepresentative enzymes

^dSubgroup requiring calcium ions for activity are called calpains

^eMostly exopeptidases

^fRarely, the catalytic ion is Ni^{2+} or Co^{2+} (not in humans)

(Adapted from Table 1 in Puente et al. "Human and mouse proteases: a comparative genomic approach." *Nat. Rev. Genet.* 4(7):544–558, 2003 and updated according to Puente et al., "A genomic view of the complexity of mammalian proteolytic systems." *Biochem Soc Trans.* 33 (Part 2):331–334, 2005)

Table 8.2 Types of zincin metalloproteases in humans

Type of enzyme	Action	Typical enzyme	Structural class
Exoproteinase	Removes N-terminal amino acid	Alanyl Aminopeptidase ^a	Metzincin
Exoproteinase	Removes C-terminal amino acid	Carboxypeptidase A ^b	Gluzincin
Peptidase	Removes a C-terminal dipeptide	Angiotensin-converting enzyme ^c	Gluzincin
Peptidase	Cuts a peptide (<13 aa) internally	Neurolysin ^d	Gluzincin
Endoproteinase	Cuts a large polypeptide internally	Matrilysins, adamalysins and astacins ^e	Metzincin

^aAminopeptidase N (APN or CD13) is expressed in many cells, tissues, and species. It cleaves the N-terminal amino acids from bioactive peptides, leading to their inactivation or degradation. It has putative involvement in antigen processing and presentation, cell adhesion, tumor invasion and metastasis, neurotransmitter degradation, and as a coronavirus receptor

^bCarboxypeptidase is a digestive enzyme. It is synthesized in the pancreas, secreted into the small intestine, and hydrolyzes the C-terminal end of proteins and peptides

^cAngiotensin-converting enzyme (ACE) cleaves dipeptides from the C-terminus of oligopeptides, notably converting angiotensin I (eight amino acids) to angiotensin II (six amino acids); angiotensin converting enzyme (ACE). The product of enzyme activity causes an increase in blood pressure

^dNeurolysin acts only on substrates of less than about 19 amino acid residues (oligopeptides), with a particular preference for cleaving near the C-terminus. Neurolysin is known by many other names, including oligopeptidase M and soluble angiotensin II-binding protein

^eMajor metalloprotease families in humans (see text)

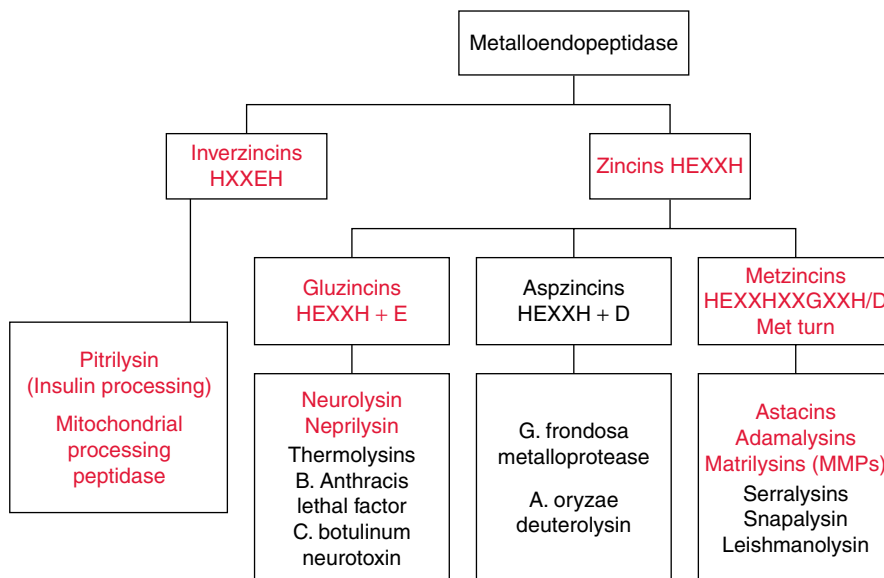


Fig. 8.1 Classification of metallopeptidases by zinc-binding motifs. The major amino acid motif in zincins has two histidine residues that coordinate with the metal ion and a glutamate residue (E) for catalysis. A third residue that coordinates with the metal may be glutamate, aspartate (D), or histidine. In metzincins, the third coordinating residue is histidine or aspartate (H/D), but the name is taken from the presence of a downstream invariant methionine residue (see Fig. 8.2 and text). The *red type* indicates enzymes or enzyme subfamilies encoded in the human genome (Slightly modified from Fig. 1A of F.X. Gomis-Ruth, “Structural aspects of the metzincin clan of metalloendopeptidases.” Mol. Biotechnol. 24(2):157–202, 2003)

astacins (Fig. 8.1), are involved in procollagen processing. Metzincin classes not encoded in the human genome are mostly bacterial virulence factors such as anthrax lethal toxin, a serralyisin. Additional metzincins have recently been identified in plants and bacteria.

8.1.2.

Catalytic Action of the Metzincin Family

The metzincin catalytic domain consists of a flat surface within a small cleft within which peptide substrates bind and are hydrolyzed (Fig. 8.2a). In astacins, the catalytic domain is stable, but adamalysins require a calcium ion to stabilize the flat surface of the domain above the cleft. Matrilysins require *two* calcium ions and a second, *noncatalytic zinc* ion to stabilize this domain (Fig. 8.3). Table 8.3 reviews the roles of specific metal ions that participate in the various stages of collagen processing discussed in this chapter and Chap. 7.

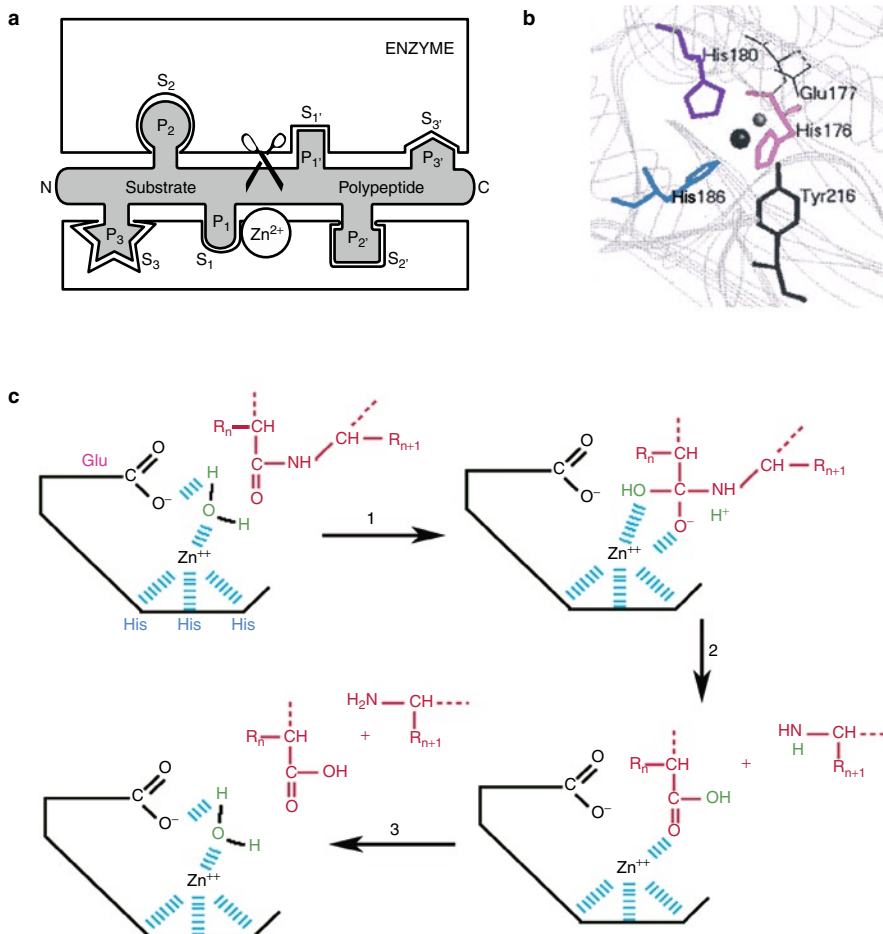


Fig. 8.2 Catalytic metzincin domain. **(a)** Catalytic endopeptidase subunit showing the peptide binding cleft and surrounding domains. The site binds six amino acids on either side of the bond to be hydrolyzed. Substrate is in an extended conformation and the bound amino acids are numbered (N- to C-terminus): ... $S^3-S^2-S^1-S^1'-S^2'-S^3'$... The products are two peptides: ... $P^3-P^2-P^1-COOH$ and $NH_2-P^1'-P^2'-P^3'$... (Slightly modified from Fig. 1B of F.X. Gomis-Ruth, "Structural aspects of the metzincin clan of metalloendopeptidases." *Mol. Biotechnol.* 24(2):157-202, 2003). **(b)** Active site crevice of the metzincin catalytic domain from above. The conserved zinc-binding motif has the structure: His¹⁷⁶-Glu¹⁷⁷-Xaa-Xaa-His¹⁸⁰-Xaa-Xaa-Gly-Xaa-Xaa-His¹⁸⁶. The numbering is for serralsin, an astacin family protein, but the relative positions of these amino acids within the motif are identical in all metzincins. The catalytic zinc ion (*large sphere*) has a coordinated water molecule (*small sphere*) whose hydrogen atoms are H-bonded to Glu¹⁷⁷ (bond shown in Fig. 8.2c). About 20 residues, downstream lies a tyrosine phenolic side chain that points back from beneath the active site as a result of a turn induced by the invariant methionine residue. The ligands involving tyrosine form a distorted trigonal bipyramid in which His¹⁷⁶, His¹⁸⁶, and the water molecule are equatorial. His¹⁸⁰ and Tyr²¹⁶, respectively, form the upper and lower axial ends (peaks of each pyramid). The Zn^{2+} ion is approximately 2.2 Å from the histidine ligands, 2 Å from the water molecule, and 2.8 Å from the phenolic O atom of Tyr²¹⁶. Adamalysins and matrilysins (and some other

The glutamate (E) residue in the common zincin motif (HEXXH) participates in catalysis by attaching a water molecule that also coordinately bonds to the catalytic zinc ion (Fig. 8.2b). A likely catalytic mechanism involves the loss of a proton from the water molecule, followed by nucleophilic attack of OH^{-1} on the peptide bond (illustrated in Fig. 8.2c). During this process in astacins such as serralsin, a tyrosine residue flips back and forth during substrate anchoring, cleavage, and product release, in a motion referred to as a “*tyrosine switch*.” The phenol group of this tyrosine coordinates with the catalytic zinc ion and displaces the water molecule, thus inhibiting catalysis. Interactions of the enzyme away from the catalytic site are transmitted to the tyrosine residue and weaken or strengthen the coordination of its phenolic group to the catalytic zinc ion. In matrilysins and adamalysins, the “*tyrosine-switch*” is replaced by a “*cysteine-switch*.” The thiol group of cysteine coordinates to the catalytic zinc ion instead of tyrosine, similarly displacing the water molecule required for catalysis. Unlike the tyrosine switch, this “off” position is virtually permanent unless the N-terminal propeptide that contains this cysteine residue is removed, allowing the thiol group to be replaced on the zinc ion by the water molecule that participates in catalysis as in astacins (Fig. 8.2).

8.1.3. Metzincin Activation

All human metzincins are secreted as proenzymes. Astacins and adamalysins are mostly activated by calcium-ion-dependent serine proteases (*pro-protein convertases*) that meet up with their substrates in trans-Golgi and secretory vacuoles. These proenzymes are known as furin-like convertases because of their homology to a serine protease called *furin* and a bacterial endoprotease called *subtilisin*. The furin-like enzymes require calcium ions to maintain structural stability whereas other serine proteases, represented by trypsin and chymotrypsin, do not. The *furin-like pro-protein convertases* autocleave their own N-terminal domain propeptide (self-activate) during secretion and then convert the N-terminal domains of co-secreted metzincins. Activation cascades also occur among the

metzincins) have proline instead of tyrosine at the turn. In these enzymes, a cysteine residue within the pro-domain blocks the cleft by binding to the catalytic zinc ion and excluding water molecule binding (see text) (Modified from Park, H.I. and Ming, L.J. “Mechanistic studies of the astacin-like Serratia metalloendopeptidase serralsin: highly active (>2,000%) Co(II) and Cu(II) derivatives for further corroboration of a “metallotriad” mechanism.” J. Biol. Inorg. Chem. 7(6):600–610. 2002). (c) *Mechanism of catalysis*. (1) The tyrosine residue moves away from the zinc ion and the water molecule (*green*) attacks the substrate polypeptide (*red*) under the influence of the deprotonated glutamate residue. (2) The C-terminal peptide amino group picks up the proton and is released. (3) The remaining hydroxide anion attacks the carboxyl group, which remains held by the zinc ion but it is quickly replaced by another water molecule (Modified from University of Tours, France Web site: <http://delphi.phys.univ-tours.fr/Prolysis/introprotease.html>)

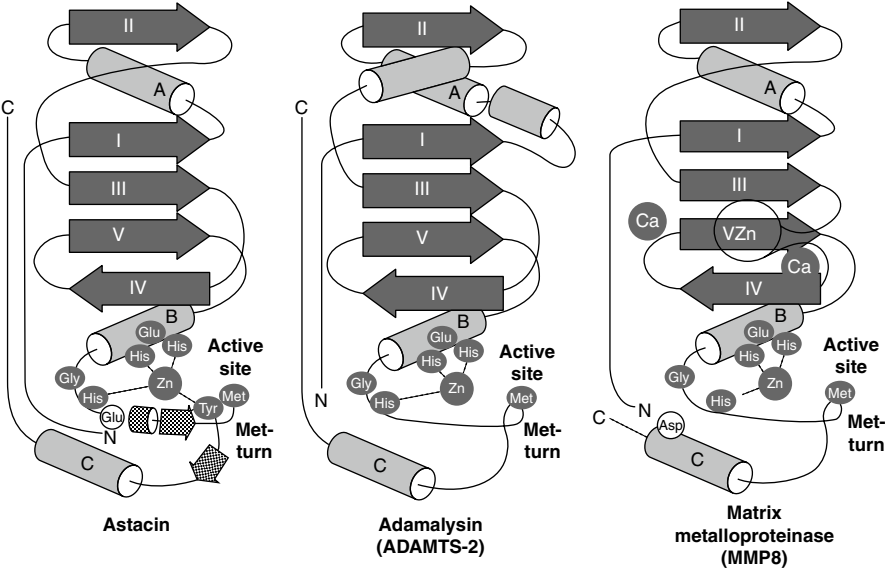


Fig. 8.3 Architecture of astacins, adamalysin type-2, and matrix metalloproteinases. Topology scheme of astacins, adamalysin type-2, and matrix metalloproteinases. The α -helices are shown as rods, β -sheet strands as *arrows*, and unstructured regions as *thin lines*. Key amino acids in the catalytic, zinc-binding motif are shown (*white in black ellipse*). Distinguishing features of each structure are shown as *lighter gray* for amino acids (*black letter*), and as unlabeled, secondary-structure elements. In addition to the catalytic zinc ion in all three structures, the calcium ion in adamalysin type-2, and the two calcium ions and second zinc ion in the matrix metalloproteinases (Abbreviated from Fig. 3 in F.X. Gomis-Ruth, “Structural aspects of the metzincin clan of metalloendopeptidases.” Mol. Biotechnol. 24(2):157–202, 2003)

Table 8.3 Metal ions required for collagen processing

Metal Ion/s	Enzyme/s
Fe^{2+}	Proline/lysine hydroxylase
Cu^{1+}	Lysyl oxidase
$\text{Mg}^{2+}/\text{Mn}^{2+}$	Integrin attachment of collagen to cells
$\text{Zn}^{2+}/\text{Ca}^{2+}$	Metalloproteinases

zincins due to self- or hetero-catalysis. For example, an activated adamalysin can remove the propeptide from another adamalysin, a matrilysin or an astacin, all independently of the initiating furin-like activation.

A similar cascade is associated with inflammation following an injury, infection, or environmental stress (Fig. 8.4). It involves chymotrypsinogen-like serine proteases called

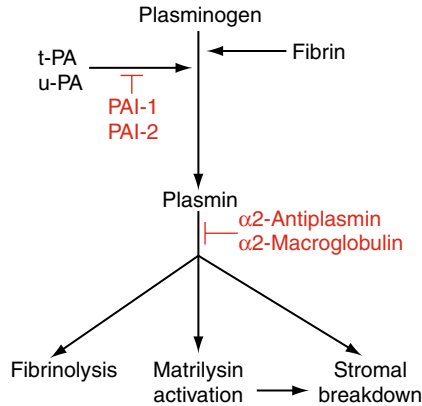


Fig. 8.4 Matrilysin activation. Pro-matrilysins are activated by *plasmin*. This serine protease does not require Ca^{2+} for activation, unlike *furin*, which activates the procollagen peptidases (Fig. 8.5). Plasmin is obtained from a precursor, *plasminogen*, by plasminogen activators. Plasminogen is made in the liver and secreted into the blood plasma. *Plasminogen activators* are secreted in the tissues as urokinase related, u-PA, or as a tissue type, t-PA. tPA is made by fibroblasts and basal epithelial cells. It is present in small amounts in the stroma where it activates plasminogen released from injured capillaries and causes fibrinolysis (Sect. 11.4.2) (Copy of Fig. 1 from B. Kinnby, “The plasminogen activating system in periodontal health and disease”. Biol. Chem. 383:85–92, January 2002; copyright permission given by Walter de Gruyter, Berlin, New York and the author)

tissue or urokinase plasminogen activators (plasminogen pro-protein convertases). Plasminogen is made in the liver, secreted into the blood plasma, and activated its single polypeptide chain being cut into two disulfide linked chains, like the cutting of prothrombin by factor Xa (Sect. 11.3.4.). If plasminogen escapes from capillaries, it is converted by a plasminogen activator enzyme in the tissues (usually t-PA, Fig. 8.4) to plasmin. Plasmin degrades fibrin blood clots (Chap. 11). In the stroma, plasmin also cleaves fibronectin, thrombospondin (TS), laminin, and the matrilysins, especially procollagenase. These activities lyse a damaged stroma so that it can later be replaced with healthy tissue.

Excessive activation of matrilysin by plasmin is prevented by *tissue inhibitors of matrilysin proteases (TIMPs)* in a healthy stroma, or during the repair phase of inflammation (Sect. 13.2.5), or by thrombospondin-2. (Sect. 3.2.2), or during the repair phase of inflammation (Sect. 13.2.5). The N-terminal domains of one of four homologous TIMPs bind tightly and irreversibly to the catalytic site of activated matrilysins and adamalysins. Tissue damage must be maintained at sufficiently high levels for enough plasmin to have been activated before these inhibitors are significantly depleted along with the matrix. Uncontrolled matrilysin activation characterizes many chronic diseases including periodontal disease (Sect. 13.3.1).

Collagen processing and degradation are accomplished by zinc-containing metalloendoproteinases (zincins) that cleave polypeptides into large fragments. The catalytic zinc ion is coordinated to two histidine residues in a motif (HEXXH) and subdivided by a third zinc-coordinated residue: glutamate (gluzincins), aspartate (aspzincins), or histidine or aspartate associated with a downstream methionine-mediated fold (metzincins). Human metzincin endoproteinases are astacins, adamalysins and matrilysins. Catalysis involves a zinc-bound water molecule, the glutamate residue of the zincin motif, and an enzyme-specific recognition site. The coordinated water molecule may be displaced by a downstream phenol (tyrosine) in astacins, or thiol (cysteine) in adamalysins and matrilysins. The cysteine is part of the methionine-mediated fold, which is disconnected by activation when a large N-terminal peptide is removed by serine proteases: furin-like during development or plasminogen activators induced by stress. Stromal activation is resisted by tissue inhibitors of matrilysin proteases (TIMPs).

8.2.1. Fibrillar Procollagen Processing

Once the procollagen triple helix has assembled in the lumen of the endoplasmic reticulum (Chap. 6), it moves to the cis-Golgi cisternae in transport vesicles and then through the Golgi to the trans-Golgi, where it forms bundles before being released to secretory vacuoles (Fig. 7.1). The bundles develop as procollagen is cleaved to tropocollagen.

Collagen types I, II, III, and α_2V , all use one of the three adamalysins as procollagen N-proteases (PNPs) and one of the three astacins as procollagen C-peptidases (PCPs). The adamalysin consensus sequence is pro-gln with the hydrolytic cleavage C-terminal to the proline residue as indicated by the down pointing arrow (P↓Q) in type I collagen, ala-gln (A↓Q) in types II and III collagens, and pro-ala (P↓A) in α_2V collagen. The amino acid that follows the down-pointing arrow is the N terminal amino acid of the tropocollagen that is cleaved out by PNP. The α_1V , α_1XI , and α_2XI collagens use an astacin to remove the N-propeptide and a furin-like pro-protein convertase to remove the C-propeptide. Figure 8.5 indicates the polypeptide motifs of the nonadamalysin enzymes that cleave the various fibrillar collagens.

Furin-like pro-protein convertases activate all the procollagen processing enzymes (Fig. 8.6). Figure 8.7a shows the domain structure of the astacin family. The three common ones (grouped at the top) are bone morphogenetic protein 1 (BMP-1), mammalian tollid (mTld), and tollid like 1 protein (TLL-1). Protein mTld is the preferred PCP, but it cleaves slowly in secretory vesicles, preventing the bundles from growing too rapidly. Indeed, a separately secreted enhancer protein upregulates mTld activity after secretion at the cell surface, where a rapid self-assembly of collagen fibers occurs. All three astacins also process pro-lysyl oxidase (Sect. 4.2.2), the γ_2 and α_3 chains of laminin-5, and pro-biglycan, a glycosaminoglycan similar to decorin (Sect. 6.5.1) but possessing two attached glycosaminoglycans residues instead of one.

a BMP Procollagen C peptidase							
Pro- α_1 I	Tyr	Tyr	Arg	Ala	Asp	Asp	Ala
	Y	Y	R	A	D	D	A
Pro- α_2 I	Phe	Tyr	Arg	Ala	Asp	Gln	Pro
	F	Y	R	A	D	Q	P
Pro- α II	Y	M	R	A	D	Q	A
Pro- α III	P	Y	Y	G	D	E	P
Pro- α_2 V	E	F	T	E	D	Q	A

b BMP Procollagen N peptidase							
Pro- α_1 V	T	P	Q	S	Q	D	P
Pro- α_1 XI	A	A	Q	A	Q	E	P
Pro- α_2 XI	R	P	Q	N	Q	Q	P

c Furin Procollagen C peptidase							
Pro- α_1 V	R	T	R	R	N	I	D
Pro- α_1 XI	K	T	R	R	H	T	E
Pro- α_2 XI	K	T	R	R	S	V	D

Fig. 8.5 Procollagen amino acid cleavage motifs other than adamalysins. See text for adamalysin cleavage sequences. All sequences are from human procollagens. The sequences around the cleavage site for Pro- α_1 I and Pro- α_2 I are given in both three-letter and (beneath) one-letter amino acid abbreviations. The sequences for the other procollagen chains are only the one-letter abbreviations. (a) *Astacin procollagen C peptidase* (PCP). Amino acids motifs cleaved in common fibrillar collagens by PCP, the astacin bone morphogenetic protein 1 (BMP1) and the homologous enzymes (Fig. 8.6). *Bold* indicates a motif, in this case: $\phi XX\downarrow DX(A/P)$ where ϕ = hydrophobic residue; peptide bond cut = $\downarrow$ and X = any amino acid residue. (b) *Astacin procollagen N peptidase*. Amino acids motifs cleaved in type V and XI fibrillar collagens by procollagen N-protease (PNP) where PNP is BMP1 instead of adamalysin PNP (motif is $QX\downarrow QXP$). (c) *Furin procollagen C peptidase*. Amino acids motifs cleaved in above fibrillar collagens where PCP is a furin-like enzyme instead of BMP1 (motif is $BTRR\downarrow XXX$ where B is a basic residue other than R). This motif is very similar to $RXRR\downarrow$, one of two common furin consensus sequences (Sequences of all human procollagen polypeptides are public and can be downloaded from the Swiss-Protein Database. For example, Type I collagen alpha1 and alpha2 procollagen polypeptides, COL1A1 and COL1A2, are respectively at <http://www.uniprot.org/uniprot/P02452> and <http://www.uniprot.org/uniprot/P08123>). Data for type pro- α II, pro- α III, and pro- α_2 V are from C. Unsold et al., J. Biol. Chem. 277(7):596–5602. Data for pro- α_1 V, pro- α_1 XI and pro- α_2 XI are from Imamura et al., J. Biol. Chem. 273(42):27511–27517)

Note: Astacins were named from two unrelated sources. Bone morphogenetic protein 1 (BMP-1) was originally identified as a zinc metalloprotease in extracts of demineralized bovine bone together with TGF α -like growth factors (described in Chap. 3 and 8) termed BMP-2A and BMP-3. Amino acid sequencing and cDNA cloning demonstrated that mouse PCP-1 was identical to BMP-1 and that chicken PCP-2 was identical to a protein named mammalian tolloid (mTld) after a homologous *Drosophila* proteinase tolloid (TLD). BMP-1 and mTld are two of six splice variants of the *bmp1* gene. Two related genes encoding proteases similar to mTld have been identified in *bmp1* null mice: mammalian tolloid like-1 and -2 (mTLL-1 and mTLL-2). The *bmp1* null mice make abnormal collagen fibrils in the skin and fail to close the ventral body wall. Homozygous loss of *bmp1* is lethal in utero, but a skeleton develops because mTll-1 has PCP activity that partially compensates for BMP1.

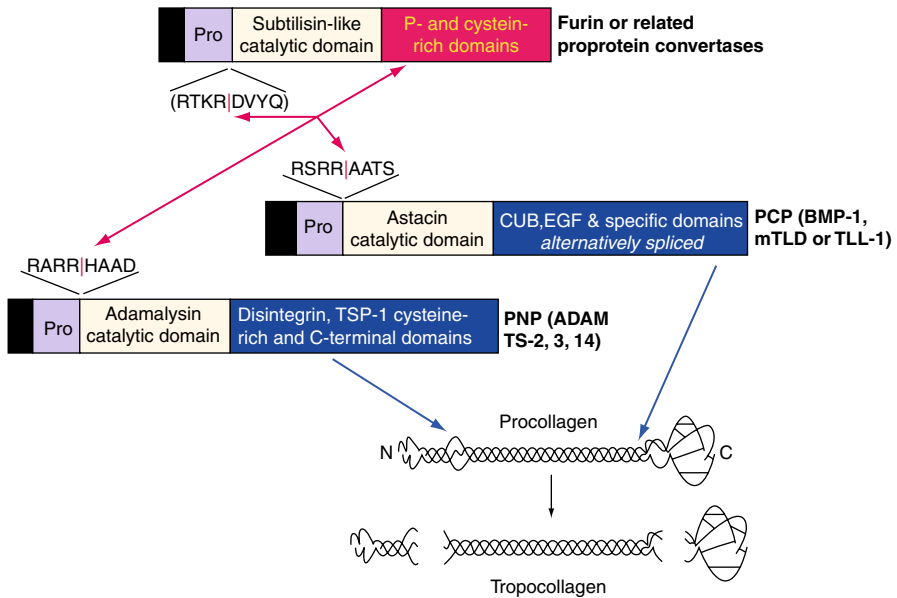


Fig. 8.6 Activation of procollagen N- and C-proteinases. The signal sequence for secretory targeting is represented as a *black rectangle* and is removed in the endoplasmic reticulum prior to secretion. Each of the C-terminal domains is labeled in the figure. The activator pro-protein convertases furin, procollagen C-peptidase (PCP) and procollagen N-protease (PNP) each contain a pro-domain (Pro; purple) that ensures proper folding. The pro-domain of furin is auto-catalytically removed, but the pro-domains of PNP and PCP are not. The first domain of furin after its signal- and pro-domains is the protease domain, related to the subtilase superfamily of Ca^{2+} -dependent proteases. Similarly, the first domain of PCP and PNP after their signal and pro-domains is a metalloendoprotease. Following the catalytic domain of each protein is a mixture of C-terminal domains. In furin and other pro-protein convertases, the so-called P-domain is essential for activity C-terminal extensions for correct localization. The C-terminal domains of the PCPs modulate activity, whereas those of PNP specify collagen-type substrate (Modified slightly from Fig. 4 in E.C. Canty and K.E. Kadler. "Procollagen Trafficking, Processing, and Fibrillogenesis." J. Cell Sci. Vol. 118(7):1341–1353, 2005; Reproduced with permission of the Company of Biologists)

Removal of the less bulky N-propeptide of procollagen proceeds similarly. The released collagen N-terminal propeptide is reabsorbed back into the cytosol where it inhibits collagen translation and prevents excessive fiber formation (feedback inhibition). The most common PNP is an *adamalysin of the thrombospondin type-2* class (ADAMTS-2), which is structurally large and complex, containing nine domains (Fig. 8.7b). The pro-domain is essential for correct folding during polypeptide synthesis. The thrombospondin (TS) domains through the C-terminus of the protein specify the proper orientation of enzyme binding for catalysis. Replacing the three C-terminal TS repeats with those in a closely related protein, ADAMTS-14 (PNP for type $\alpha_2\text{V}$ collagen), prevents all enzymatic activity toward type I collagen, whereas removing only the C-terminal domain enhances the type I collagen activity.

Snake venoms cause a rapid disintegration of the stroma (*disintegrin*) due to short peptides each containing an RGD integrin-binding sequence. The RGD sequence displaces

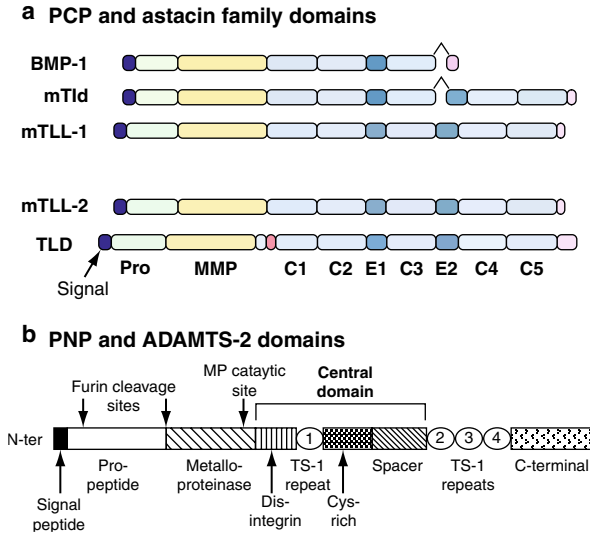


Fig. 8.7 Domain organization of the two procollagen peptidases. **(a)** *Domain organization of procollagen C-peptidase (PCP) and related astacins.* Signal peptides are dark purple, prodomains are green, proteinase domains are yellow, CUB domains are light blue, EGF-like domains are light purple, and domains unique to each protein are shown in pink. The CUB domains are labeled C1 through C5 and the EGF domains are labeled E1 and E2. CUB domains are present in functionally diverse, mostly developmentally regulated proteins and also in peptidases belonging to astacin and chymotrypsin families. It is an extracellular domain of approximately 110 amino acid residues containing four conserved cysteines that form adjacent disulfide bridges. The CUB domain is predicted to have a beta-barrel structure similar to that of immunoglobulins (antibody molecules). The EGF domain does not include the N-terminal Ca^{2+} -binding subdomain discussed in relation to fibrillin (Sect. 6.1.1) (Slightly modified from Fig. 1 in G. Ge. and D.S. Greenspan, *Developmental Roles of the BMP1/TLD Metalloproteinases*. Birth Defects Research (Part C), 78:47–68, 2006). **(b)** *Domain organization of procollagen N-peptidase, ADAMTS-2 adamalysin.* The four thrombospondin type 1 (TS-1) repeats (circles) are numbered as indicated Thrombospondins are discussed in Sect. 3.2.2). ADAMTS-3 and ADAMTS-14 proteins are homologous (Slightly modified from Fig. 1 in A. Colige et al. “Domains and maturation processes that regulate the activity of ADAMTS-2, a metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types I-III and V.” *J. Biol. Chem.* 280(41):34397–34408, 2005)

adamalysins from integrins on the cell surface (Sect. 3.2.1). The released adamalysins float freely in the stroma and behave like activated nonspecific matrilysins. Integrin-bound metalloproteases are critical for ovum fertilization, and so the name of this group of proteins was cleverly transformed into a biochemical name: A Disintegrin And Metalloprotease Domain (ADAM), or ADAMalysin. The ADAMTS-2 proteins comprise a subfamily of adamalysins possessing thrombospondin domains. The ADAM family proper consists of over 40 proteins, one of which is described in Chap. 13 (Sect. 13.2.2). These latter proteins possess a canonical disintegrin domain that keeps them integrin-bound at the outer cell surface. A different disintegrin domain in the ADAMTS family enables their secretion instead of remaining cell surface bound.

Procollagen N-terminal processing is performed mostly by adamalysins and C-terminal processing mostly by astacins. Processing begins with procollagen bundle formation. Some cleavage of all three polypeptides at mostly the N-terminus occurs within the secretory vacuole, but secretion activates rapid C-terminal cleavage and spontaneous fiber formation. The adamalysin responsible for procollagen N-terminal processing is held to integrins at the cell surface following secretion and this also promotes faster extracellular procollagen processing to tropocollagen.

8.3.1.

Matrilysins (MMPs) Hydrolyze Collagen and Stromal Proteins

Matrilysins (MMPs) are required for stromal remodeling during development, pregnancy, and growth, and also following trauma or infection. Different classes degrade different extracellular matrix protein components: fibers, anchoring and basement membrane collagens, proteoglycans, laminin, fibronectin, and other stromal proteins. Many also participate in proteolytic events required to control diverse physiological processes: cell surface release of growth factors, activation of cytokines and receptors, and the inactivation of proteinase and angiogenesis inhibitors.

As noted in the previous section, matrilysin catalysis is held in check by endogenous tissue inhibitors of metalloproteinases (TIMPs), which irreversibly bind to the active site. Different TIMPs first bind to hemopexin-like domains on almost all matrilysins before they can bind to the active site, thus providing some TIMP specificity. Hemopexin is a plasma protein that binds to heme and transports it to the liver for conversion to bile. It is formed by the repetition of a variable length unit of 35 to 45 residues, the hemopexin-like domain. TIMPs are the ligands for a modified, homologous hemopexin-like domain on matrilysins. Figure 8.8 diagrams the structures of matrilysins most relevant to the topics in this book.

There are at least 28 matrilysins that participate in connective tissue degradation as collagenases, gelatinases, elastases, and stromelysins. All 28 matrilysin enzymes are listed in Table 8.4 along with their matrilysin (MMP) number and cell expression. The molecular weights of the most relevant pro- and activated enzymes, and their substrate specificities, are listed in Table 8.5. Collagen is primarily degraded by MMP-1 and -8 (fibroblast and neutrophilic granulocyte collagenase) and MMP-2 and -9 (fibroblast and neutrophilic granulocyte gelatinase). Fibroblast gelatinase (MMP-2) secretion is inhibited by thrombospondin-2, causing excessive collagen synthesis (fibrosis), which not only limits the spread of an infection, but also destroys tissue architecture and causes implants rejection (foreign body reaction; Sect. 3.2.2).

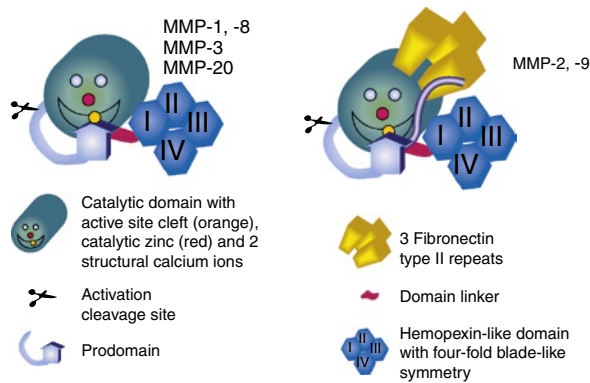


Fig. 8.8 The domain arrangement of the matrilysins. *White dots* represent two calcium ions that contribute to the structural integrity of the zincin catalytic domain. The *red dot* represents the zinc ion, the yellow dot represents the active site cleft with substrate binding sites represented by the ‘smile’. The thick light-blue arrow represents the prodomain in the binding cleft and the scissors represent where the prodomain is removed by plasmin. Figure is composed of the right top two parts of Fig. 1 in W. Bode and K. Maskos, Structural basis of the matrix metalloproteinases and their physiological inhibitors, the tissue inhibitors of metalloproteinases. Biol. Chem. 384 (June):863–872, 2003; Copyright permission given by Walter de Gruyter, Berlin, New York and both authors

Table 8.4 Cellular sources of matrix metalloproteinases

Protease class	MMP number	Keratinocyte/leukocyte expression
Collagenases	MMP-1 ^a , 8 and 13	Keratinocyte ^b : MMP-1, 3, 9, 10, and 28
Gelatinases	MMP-2 ^a and 9	Granulocytes ^c : MMP-8, 9, and 25
Stromelysins	MMP-3 and 10	B Cells ^d : MMP-11, 26, and 27
Membrane-type MMPs	MT-MMPs 14 ^a – 17; 24, and 25	T cells ^c : MMP-15,16, 24, and 28
Others	MMP-7, 11, 12, 19, 20, 21, 23, 26, 27, and 28	Monocytes ^f : MMP-1, 2, 3, 9, 10, 14, 17, 19, and 25

^aMajor MMPs expressed by fibroblasts

^bExpression enhanced in proliferative/migratory basal keratinocytes at wound edge. Around ulcerations of mucosal tissues, such as lung and intestine, MMP-28 (epilysin) is absent but MMP-7 (matrilysin - from which all this group of MMPs takes its name.) is present instead MMP-7 degrades most major non-collagen proteins in a stromal matrix

^cMostly in neutrophilic granulocytes attracted to a region of stromal injury

^dAntibody-producing lymphocytes that become attracted to a site of infection

^eNon-antibody producing lymphocytes also attracted to a site of infection

^fMacrophage precursors that develop from the white blood cell infiltrates at sites of stromal injury or infection

Table 8.5 Matrilysin connective tissue degrading enzyme specificities

MMP#	Enzyme name	Molecular mass, latent (kDa)	Molecular mass, active (kDa)	Substrates
MMP-1	Collagenase-1 (fibroblasts)	55	45	Fibrillar collagens, gelatin, proteoglycans
MMP-8	Collagenase-2 (neutrophils)	75	58	Fibrillar collagens
MMP-13	Collagenase-3 (many cells)	65	55	Collagen type II
MMP-2	Gelatinase A (fibroblasts)	72	66	Gelatin, collagen type IV, elastin, fibronectin
MMP-9	Gelatinase B (neutrophils)	92	86	Gelatin, collagen type IV, elastin
MMP-7	Matrilysin	28	19	Matrix components except fibrillar collagens
MMP-3	Stromelysin-1	57	45	All matrix components except elastin and fibrillar collagen
MMP-10	Stromelysin-2	57	44	Matrilysin without elastase or laminin activity
MMP-11	Stromelysin-3	59	44	Laminin
MMP-12	Metalloelastase	53	45/22	Elastin, fibronectin, collagen type IV
MMP-20	Enamelysin	54	43	Amelogenin

8.3.2.
Stromelysins

Stromelysins-1, -2, and -3 (MMP-3, MMP10, and MMP-11) degrade stromal components other than collagen. Skin fibroblasts constitutively express progelatinase (MMP-2), and activate it by co-secreting a membrane-adherent matrilysin on their cell surface, especially MMP-14. In contrast, following exogenous stresses or exposure to cytokines and ultraviolet irradiation, fibroblasts secrete procollagenase (MMP-1) and neutrophils secrete progelatinase (MMP-9). These enzymes are activated by plasmin from stress-activated *plasminogen pro-protein convertases* (see Sect. 8.1.3).

8.3.3.
Enamelysin

Enamelysin (MMP-20) has major domains and an overall structure identical to fibroblast collagenase, gelatinase, and some stromelysins, but it lacks conserved residues that

determine collagenase or stromelysin specificity. A shorter amino acid sequence of the catalytic domain's C-terminal region further distinguishes it from collagenase and gelatinase. Enamelysin cleaves amelogenin, a major protein that determines enamel crystallization (Chap. 9). Except for collagenases (Sect. 8.3.4), the roles of the remaining matrilyns are not yet known.

8.3.4.
Collagenases and Gelatinases

Collagenases act on collagen fibers at neutral pH. They recognize a three-dimensional structure that recurs at the gaps in the quarter-staggered array of tropocollagen molecules and cleave all three polypeptides at that point. This cut (Fig. 8.9a) causes the tropocollagen triple helix to spontaneously unwind, exposing individual one-quarter and three-quarter

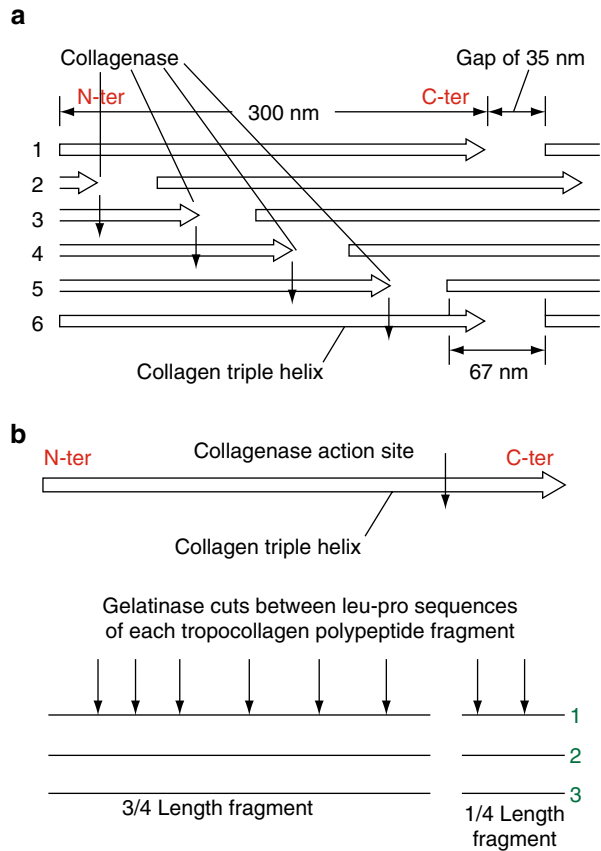


Fig. 8.9 Modes of action of neutral collagenase and gelatinase on collagen fiber. **(a)** *Initial step:* Collagen degradation begins with neutral collagenase cutting a triple helix into N-ter $\frac{3}{4}$ and C-ter $\frac{1}{4}$ fragments. **(b)** *Final step:* The $\frac{3}{4}$ and $\frac{1}{4}$ -length tropocollagen α -chain fragments unwind, exposing its leu-pro bonds to gelatinase and it is degraded to small peptides

length polypeptides to gelatinase. The gelatinase cleaves exposed leucine–proline bonds, which are common in the tropocollagen sequence (Fig. 8.9b). The resultant small peptides are taken up by local cells and degraded to free amino acids in their lysosomal vesicles. Excessive collagen fiber cross-linking slows the unwinding of the three chains and their rate of degradation is slowed considerably. Collagen fibers are therefore difficult to turn over in old age because of extensive cross-linking. (Sect. 4.2.2), resulting in tissue malfunctions associated with senescence.

Collagenase and gelatinase are produced by fibroblasts and neutrophils. Although catalytically identical, the respective cells utilize different genes with homologous but non-identical amino acid sequences (Table 8.5). The fibroblast enzymes are larger and produced in a different environment from the neutrophil enzyme. Fibroblast gelatinase cleaves monocyte chemoattractant protein-3 (MCP-3), which prevents leukocyte infiltration of developing or remodeling tissues. The MCP-3 cleavage products bind to and inhibit a monocyte receptor that intact MCP-3 activates on monocytes and neutrophils. MCP3 is cleaved because it binds to the hemopexin domain of fibroblast gelatinase (MMP-2), but not to the corresponding domain of neutrophil gelatinase (MMP-9). Neutrophilic granulocytes are absent during development, but present in large numbers following tissue damage or infection when intact MCP3 actively recruits granulocytes to the affected region (Sect. 13.3.1).

There are 28 matrilysins that degrade various stromal proteins, most importantly fibrous collagen: MMP-1 and -8 (collagenases) and MMP-2 and -9 (gelatinases). Collagenases cleave all three tropocollagen polypeptides into large N-terminal and small C-terminal fragments that spontaneously unwind, exposing leu-pro bonds. Gelatinase cleaves these bonds to short peptides that are endocytosed and digested to amino acids in lysosomal vesicles. The collagenases and gelatinases are expressed by fibroblasts and neutrophils, respectively. They are separately encoded: fibroblast gelatinase will hydrolyze monocyte chemoattractant protein-3 (MCP-3), preventing inflammation during development when neutrophils are absent. Neutrophil gelatinase cannot cleave MCP-3.

Mineralization is the precipitation of calcium phosphate, but biochemical mediation of this process is not fully understood. In this chapter, the chemistry underlying mineralization (Sect. 1) and the structures of bones and teeth (Sect. 2) are described. Osteoblasts secrete osteoid matrix and matrix vesicles that transport type I collagen and calcium phosphate, respectively, to the matrix where they will mineralize. Secreted matrix vesicles take up calcium and phosphate until they burst and release the calcium phosphate, which then redissolves and remineralizes around the type I collagen (Sect. 3). Glycoproteins involved in correctly modeling bone and dentin, and the role of osteocalcin in limiting excessive bone growth is then discussed (Sect. 4). There follows a detailed description of enamel (E) mineralization and of the major proteins involved (Sect. 5) followed by two summaries: the difference between enamel and bone mineralization, and the vitamins required for mineralization (Sect. 6).

9.1.1.

Fundamental Properties of Calcium Phosphate Precipitation

Calcium ions precipitate with phosphate ions where their dissolved free ion activities (concentrations at low ionic strength) exceed their solubility product – the product of the molar concentrations of each ion powered to its respective charge. Calcium is invariably present as a divalent ion (Ca^{2+}), but phosphate ions assume one of three, pH-dependent forms (Fig. 9.1a, left): dihydrogen phosphate ($\text{H}_2\text{PO}_4^{1-}$), monohydrogen phosphate (HPO_4^{2-}), and phosphate (PO_4^{3-}). In solution above pH 6.2, a predominance of calcium dihydrogen phosphate transitions to a predominance of calcium monohydrogen phosphate. Calcium monohydrogen phosphate (solubility product $\sim 1 \times 10^{-6}$) is about 100 times less soluble than calcium dihydrogen phosphate (solubility product $\sim 1 \times 10^{-4}$). Precipitated calcium phosphate is commonly referred to as apatite.

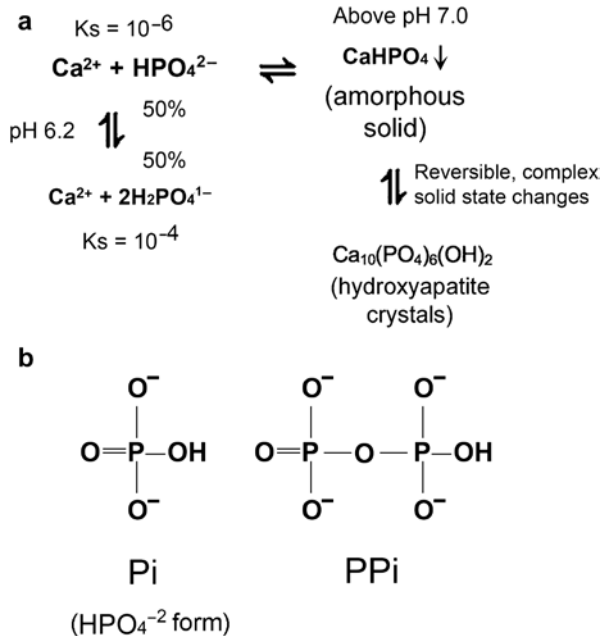


Fig. 9.1 Calcium phosphate precipitation and structures of orthophosphate (Pi) and pyrophosphate (PPi) ions. **(a)** *Calcium phosphate precipitation and solubility at different pH.* Left side of equation summarizes that, at pH 6.2, the fraction of monohydrogen and dihydrogen phosphate ions is about equal and that calcium dihydrogen phosphate is 100 times less soluble than calcium monohydrogen phosphate. *Right side* of the figure shows that phosphate ions exist mostly as monohydrogen phosphate at pH 7. Calcium monohydrogen phosphate precipitates as an amorphous solid that spontaneously rearranges to form hydroxyapatite crystals. **(b)** *Structures of Pi and PPi.* Structures are the ionized forms at physiological pH (~7.2). (Original figures)

9.1.2.

Nature of the Apatite Precipitate

The apatite that initially precipitates is crumbly and brittle due to its amorphous (noncrystalline) structure. If the surrounding fluid remains above pH 7, this apatite undergoes a series of spontaneous, solid-state rearrangements whose major end product is hydroxyapatite, a crystal containing ten calcium ions, six phosphate (PO_4^{3-}) ions, and two hydroxide (OH^{1-}) ions. These changes are due to a spontaneous alkalinization of the apatite, in which monohydrogen phosphate (HPO_4^{2-}) ions lose a proton and hydroxide ions appear from water molecules trapped in the initial amorphous precipitate (Fig. 9.1a, right). Hydroxyapatite is a long, thin, flat crystal that forms a thick, hard, flat surface that is primarily responsible for the strength of bones and teeth. The long axis is referred to as the “c” axis. The width is the “b” axis and the thickness is the “a” axis.

During bone formation, the crystals form with their “c” axis parallel to the collagen fiber and thicken by accretion at their “a” and “b” axes. During enamel formation, apatite crystals join end-to-end at their “c” axis, forming thin ribbons that become enamel rods.

9.1.3.

Apatite Crystal Substitutions Influence Bone Strength and Solubility

The hydroxyapatite crystals in bone and teeth are imperfect due to other anions and cations, especially magnesium, chloride, carbonate, and fluoride ions. Carbonate (CO_3^{2-}) is the most important. At low carbonate contents (<4% by weight), a carbonate ion replaces a phosphate ion in the crystal (“A” site substitution), but at higher contents (>4% by weight) it replaces a hydroxide ion (“B” site substitution). Either substitution slightly shortens and fattens the crystal (“c” or “a” axes increase) and increases solubility. In contrast, if hydroxide ions are present, they can be replaced by fluoride, which decreases apatite solubility (Sect. 16.2.1). Crystallographic analyses indicate that, in bone and dentin, phosphate is often replaced by carbonate, whereas in enamel it is more often replaced with chloride (Cl^-). Carbonated hydroxyapatite is critical for enamel development (see Sect. 9.5.3).

Enamel mineral has many large hydroxyapatite crystals, whereas bone has many small ones with numerous vacancies and substitutions. These differences increase the elasticity of bone compared with enamel and promote its interactions with the surrounding collagen. Recently, a tightly bound “hydration shell” that fills a porous collagen-apatite junction was discovered around normal bone crystals. The water-filled pores are normally immobile, but repeated stresses cause the water to leak out from between the mineral and collagen. The drying increases mineralization and crystal formation, which may explain the decreased elasticity of bones with age.

9.1.4.

Nucleation

Dissolved calcium and phosphate ions may remain soluble despite their concentrations exceeding the solubility product in blood plasma and stromal extracellular (interstitial) fluid where the pH is just above 7 (Sect. 3.3.1). In blood plasma, mineralization is prevented by polyanions, especially albumin, citrate, and pyrophosphate (PPi), which chelate calcium ions and prevent their precipitation with monohydrogen phosphate ions (*orthophosphate*, Pi , or HPO_4^{2-}). *Pyrophosphate (PPi)* inhibits the premature aggregation of calcium with monohydrogen phosphate ions in mineralizing tissues and interstitial fluid throughout the body (Fig. 9.1b).

For mineralization, the normal, metastable state is adjusted by *nucleation*, measured by the seed and solubility tests. The *seed test* measures amount of solid apatite required to precipitate Ca^{2+} and HPO_4^{2-} ion concentrations exceeding their solubility product. The *solubility test* measures the minimal concentrations of Ca^{2+} and HPO_4^{2-} necessary to induce precipitation. Type I collagen fibers nucleate bone formation as the concentrations of Ca^{2+}

and HPO_4^{2-} ions increase. Premature nucleation is prevented by pyrophosphate (PPi; Fig. 9.1b), small amounts of which strongly inhibit nucleation.

PPi is made in three ways: (1) in the nucleus as a by-product of RNA synthesis ($\text{nNTP} \rightarrow \text{NMP}_n + \text{nPPi}$); (2) in the cytosol as a by-product of amino acid activation for protein synthesis ($\text{aa} + \text{ATP} \rightarrow \text{aaAMP} + \text{PPi}$); and (3) by acetyl CoA synthetase on the outer mitochondrial membrane prior to its degradation for ATP production ($\text{R-COOH} + \text{ATP} + \text{HS-CoA} \rightarrow \text{R-Co-SCoA} + \text{AMP} + \text{PPi}$). Amino acid activation is the major source of cytosolic PPi, which is transported by the ANK protein to the osteoid matrix to inhibit premature mineralization (see Sect. 9.3.5).

Mineralization is the precipitation of calcium and phosphate ions above pH 7. Initial precipitates are soft and noncrystalline (amorphous). If left alone, a solid-state rearrangement slowly and spontaneously forms hydroxyapatite, whose crystals each contain ten calcium (Ca^{2+}), six phosphate (PO_4^{3-}), and two hydroxide (OH^{1-}) ions. The resulting hard, flat surface is primarily responsible for the strength of bones and teeth. Substituting hydroxide or phosphate ions with carbonate ions increases crystal solubility, whereas substituting hydroxide ions with fluoride ions decreases crystal solubility. In bone, hydroxyapatite crystals have many spaces and substitutions, permitting a water layer between apatite and collagen that dries up and decreases bone's elasticity with age. Biological fluids are supersaturated with calcium and phosphate, but contain pyrophosphate and polyanions that inhibit spontaneous precipitation. Pyrophosphate interferes with calcium phosphate aggregation and polyanions (citrate, albumin, and other negatively charged proteins) chelate calcium ions and prevent them from being free in solution to precipitate.

9.2.1. The Structures of Bone, Dentin, and Cementum

There are two types of bone tissue: dense (*compact* or *cortical*) and spongy (*cancellous* or *trabecular*). The difference lies in how tightly the tissue is packed together. Bone matrix is predominantly a mixture of type I collagen fibrils, that resist pulling forces, and calcium phosphate mineral (apatite crystals) that resist compression. The volumes of collagen and mineral in bone are about equal, but the collagen accounts for only ~20% of the bone weight.

Compact bone consists of closely packed osteons (Haversian systems). In these osteons, central canals called the osteonic (Haversian) canals are surrounded by concentric rings (lamellae) of calcified matrix (Fig. 9.2). Bone cells (osteocytes) lie between the calcified rings, in spaces called lacunae. Small channels (canaliculi containing osteocyte processes) radiate from the lacunae to an osteonic (Haversian) canal to provide passageways for nutrients and excreted products. Each osteonic canal contains a central large blood capillary vessel that parallels the long axis of the bone. The capillaries are connected to each other and to larger blood vessels within a thin fibroblast-rich stroma on the surface of the bone (the *periosteum*) by mineral-perforating (Volkman's) canals.

Cancellous bone is less dense. It consists of thin plates and bars of bone (trabeculae) adjacent to small, irregular cavities (bone marrow) containing a connective tissue from

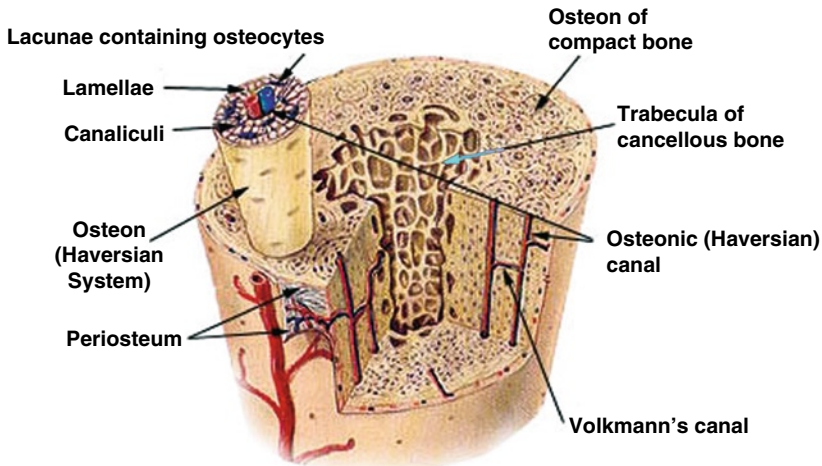


Fig. 9.2 Bone structure. Diagram of a long bone indicating the compact and cancellous structures (see text) (http://training.seer.cancer.gov/module_anatomy/unit3_2_bone_tissue.html; funded by the U.S. National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) Program, via contract number N01-CN-67006, with Emory University, Atlanta SEER Cancer Registry, Atlanta, Georgia, U.S.A.)

which the various cells that form the red and white blood cells and platelets differentiate (Sect. 11.1.1). The cavities are the equivalent of osteonic canals and are surrounded by trabeculae containing lacunae and canaliculi in which the osteocyte cell bodies and processes are respectively situated (Fig. 9.2). Although the trabeculae appear haphazard, they are in fact organized to provide maximum strength, like braces that support a building. The trabeculae of cancellous bone follow stress lines. Stress creates boney microcracks that activate osteoclasts and osteoblasts (Sect. 10.2.1), leading to appropriate remodeling and realignment. Bone cells develop as osteoblasts in the periosteum or on the surface of trabeculae and become osteocytes in lacunae following matrix mineralization. Osteoblasts and osteocytes account for about 15% of the bone mass.

Dentin is secreted unmineralized like bone. The *predentin matrix* consists of collagen, glycoproteins and proteoglycans like the osteoid matrix described below (Sect 9.3.1). The collagen fibers aggregate with their long axes parallel to long thin odontoblast processes which extend through the predentin and remain in the mineralized tissue as the center of *dental tubules*. To mineralize the dentin around the tubules, Ca^{2+} ions are transported to the mineralization front from underlying blood vessels in the developing pulp cavity. The process of mineralization is likely mediated by matrix vesicles as described for bone (Sect. 9.3.1). The innermost lining of dental tubules is mineralized last and becomes more dense than the inter-tubular dentin. The dental tubules have lateral branches that permit the odontoblasts to communicate with each other like osteocytes. These lateral branches are much more numerous in root dentin than in coronal dentin. *Unlike bone, dentin contains no blood vessels*. Cementum is deposited in layers above the dentin on the external surface of the root as a calcified matrix for the insertion of Sharpey's periodontal ligament fibers (Sect. 3.1.5). Cementum is less mineralized than compact bone or dentin.

9.2.2.

Two Mechanisms of Mineralization

Intramembranous ossification is responsible for most of the mineralization of the skull, including the maxilla and mandible. It begins with the differentiation and activation of osteoblasts from fibroblast-related precursors within a region of connective tissue that demarcates where the bone will develop. The osteoblasts secrete a nonmineralized protein-rich (osteoid) matrix and, as they move away, the matrix mineralizes (Fig. 9.3a). The periosteum remains uncalcified and contains latent and undifferentiated osteoblasts for bone remodeling. Odontoblasts (Ob) and cementoblasts secrete an osteoid-like matrix similar to that of intramembraneous ossification.

Endochondral ossification is responsible for the mineralization of long bones and it begins after chondroblasts have formed a three-dimensional cartilaginous template of the future bone (Fig. 9.3b). Blood vessels grow into the center of the cartilage and osteoblasts develop alongside invading endothelial cells at the *growth plate* where type II collagen is already present and type X collagen will develop (Sect. 4.3.2). The invading osteoblasts replace the type II and type X collagen of the endochondral growth plate with type I collagen. As the osteoid matrix is laid down, the chondrocytes proliferate and then undergo apoptosis (Fig. 9.3b). Apoptosis is described in Sect. 13.4.1. Traces of type II and type X collagen and proteo-glycosaminoglycans may remain from the cartilage and become ossified. A periosteum forms around the compact outer surface.

Bone is synthesized by osteoblasts that differentiate from assembled, mesenchymal, fibroblast-like precursors (intramembraneous ossification), or from precursors that migrate into cartilage (endochondral ossification). Bone mineralizes over an osteoid matrix composed of type I collagen fibers, which nucleate (initiate) and control the process. Outer surfaces of bone are hard (compact bone) but the insides form a cavity that is poorly mineralized (cancellous bone). The dentin and cementum of teeth resemble compact bone. The outer surfaces of bones are covered by an organic periosteum containing capillaries and an uncalcified cell-rich stroma. The central cavities also contain capillaries and a different fibroblast-like stroma within which blood cells develop. As they form, they enter the circulation where they replenish the red and white cells and platelets that mediate oxygen transport, immunity, and blood clotting.

9.3.1.

Secretion of Osteoid Matrix

Skeletal tissue mineralization (bone formation) is initiated by osteoblasts, which secrete the osteoid matrix (Fig. 9.4). They express type I procollagen in secretory vesicles together with matrix vesicles that pinch off from the membrane. The matrix vesicles are pushed away from the cell surface, possibly by the flow of fluid containing calcium and phosphate ions that are also transported through the cell from the extracellular fluid on the outer surface. Collagen fibers develop further away from the cell surface than from fibroblasts.

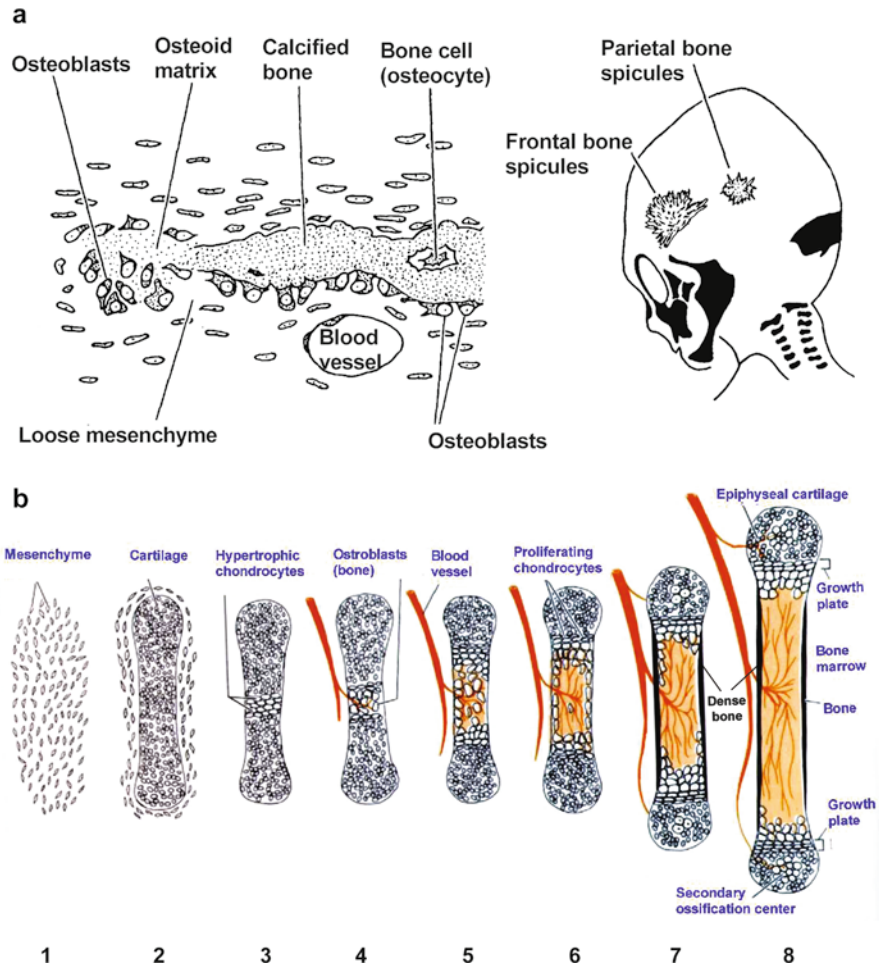


Fig.9.3 Bone synthesis. (a) *Membrane ossification is typified by cranial bone fusion.* Osteoblasts differentiate from loose mesenchymal cells resembling primitive fibroblasts. The center of cranial bones becomes cancellous as in long bones. (b) *Endochondral ossification is typified by long bone development and growth.* In this more complex process, mesenchymal cells aggregate (1) and differentiate into cartilage in the shape of the bone (2). The central region becomes hypertrophic and encapsulated (3). The lack of nutrients causes apoptosis (cell death), which attracts blood vessels and other mesenchymal cells from which osteoblasts differentiate and grow so that bone and periosteum replace the central cartilage (4). As osteoclasts resorb the enlarged central cavity (5), chondroblasts proliferate at each end (6). The periosteum induces appositional growth resembling membrane bone and the central cavity (bone marrow, red) forms cancellous bone, see text (7). At each end of the bone, the chondrocytes become apoptotic, and attract blood vessels so that secondary ossification centers develop above and below the epiphyseal cartilage and bone growth ceases (8) (Adapted from Developmental Biology, Ed. S.F. Gilbert, Sinauer Assoc. Inc., 1997 and copied from Web site <http://classes.aces.uiuc.edu/AnSci312/Bone/Bonelect.htm>)

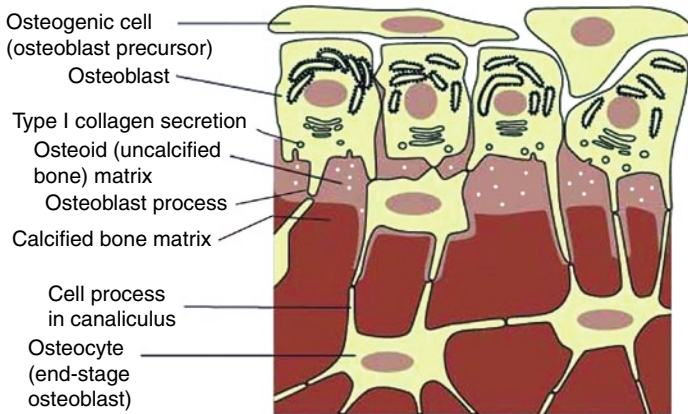


Fig. 9.4 Osteoblast secretion and matrix vesicle formation. The outer surface of all bones is covered by fibroblast-like cells that differentiate into pre-osteoblasts that secrete osteoid matrix to remodel the surface as necessary. The surface osteoblasts extend into the osteoid tissue by long processes that attach to osteocytes (fully differentiated, nondividing osteoblasts) within the bone. Changes in the environment may be sensed by the osteocytes, which transmit them as remodeling signals to the osteoblasts. The osteoid matrix is filled with many small membrane-covered matrix vesicles containing various amounts of precipitated basic calcium phosphate (*white circles*) (Modified from Fig. 22-52 in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York)

Matrix vesicles are difficult to isolate from developing membrane bone and the only well-characterized matrix vesicles available are from chondroblasts about to be replaced by osteoblasts during endochondral ossification (Fig. 9.3). The chondroblast matrix vesicles shown in Fig. 9.4 are surrounded by cartilage collagens (type II and type X) and aggrecan (Sect. 6.5.1). As osteoblasts invade and secrete their own matrix vesicles and type I collagen, the cartilage collagen and proteoglycans are almost entirely removed, presumably by matrixylins expressed by dying chondroblasts or invading osteoblasts ahead of type I collagen expression. It is not clear how cartilage derived matrix vesicles shown in Fig. 9.5 are related to osteoblast-derived matrix vesicles but they are assumed to be similar.

9.3.2.

Osteoblast Transport of Calcium and Phosphate Ions to Matrix Vesicles

Osteoblasts take up Ca^{2+} ions from the periosteal extracellular fluid using $\text{Na}^+/\text{Ca}^{2+}$ -exchangers NCX1 and NCX3. Once in the cytosol, the Ca^{2+} ions must be transported to the osteoid matrix side (basal side) by calbindins, which require the active form of vitamin D (calcitriol) for synthesis and expression. The Ca^{2+} ions are passed out to the osteoid matrix through an ATP-dependent plasma membrane Ca^{2+} -ATPase 1b (PMCA1b). The orientations of the cells, the transporters, and the calbindins are described in detail in Sect. 10.4.1. Once in the osteoid matrix, the matrix vesicles take up the Ca^{2+} ions via an *annexin transporter*.

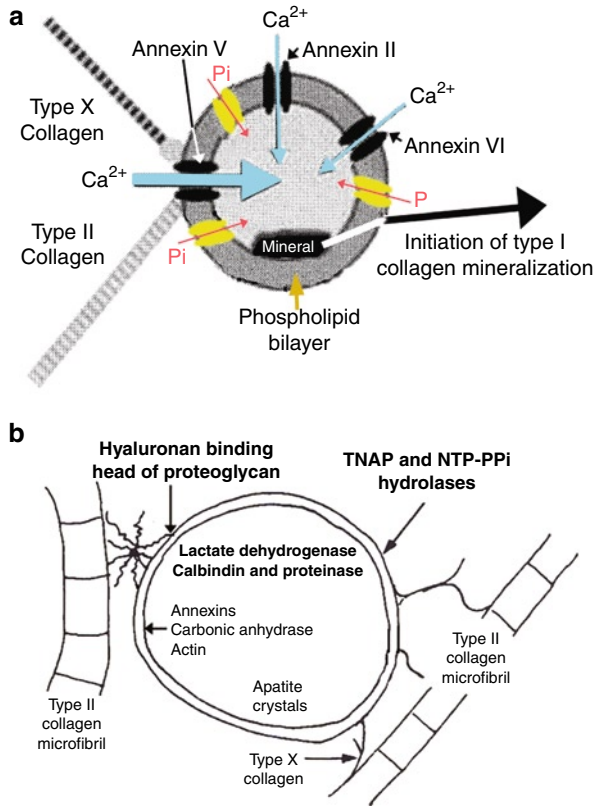


Fig. 9.5 Matrix vesicle composition. **(a) Transporters.** The major calcium transport channel is made from annexin V (thick blue arrow). Lesser amounts of annexins II and VI are also present (thin blue arrows) but their role in Ca^{2+} ion transport is uncertain. Calcium ion transport is enhanced by the presence of type II and type X collagen fibers during endochondral ossification. Phosphate transporters (PiT) are shown in yellow with a red arrow indicating the direction of phosphate transport. As each HPO_4^{2-} ion is transported into the vesicle, a sodium ion is transported out (not shown) (Modified from Fig. 5 of T. Kirsch, *Annexins and tissue mineralization: matrix vesicle, ion channel activity of annexins and annexin V/collagen interactions*, published in *Annexins: Biological importance and annexin-related pathologies* (2003). Edited by J. Bandorowicz-Pialuka, Kluwer/Plenum Publishers, 233 Spring St., New York, NY 10013). **(b) Matrix vesicle-associated enzymes and proteins.** Collagen Type X, the proteoglycan link protein and the hyaluronan binding region of proteoglycans may attach cartilage collagen (type II) to the outer surface of matrix vesicles. Nucleoside triphosphate pyrophosphohydrolase (NTAP) and NTP-PPi hydrolase are anchored in the matrix vesicle membrane. Annexin V and carbonic anhydrase are concentrated just below the matrix vesicle membrane. Lactate dehydrogenase (LDH), calbindin $\text{D}_{9\text{K}}$, and proteases are soluble in the center of the matrix vesicle (Modified to refer to osteoblast matrix vesicles from Anderson HC (1992) "Conference introduction and summary." *Bone and Mineral* 17:110)

Osteoblasts also take up orthophosphate (Pi) from the periosteal extracellular fluid using a *type I sodium/Pi co-transporter*. Pi consists of about 60% monohydrogen phosphate and 40% dihydrogen phosphate at pH 7.0. The Pi diffuses freely through the cytosol and exits

into the osteoid matrix by an unknown mechanism independently of matrix vesicle secretion. Pi then enters the matrix vesicles within the osteoid matrix through a *type III sodium/Pi co-transporter* (Fig. 9.5a) whose interior is made more alkaline by carbonic anhydrase (Fig. 9.5b) removing protons from dihydrogen phosphate by reaction with sodium bicarbonate. The carbonic acid is unstable and breaks down into water and carbon dioxide, which bubbles off and the sodium ions are exchanged to the osteoid matrix for incoming Pi. Lactic dehydrogenase is also present and it may function to prevent the pH from becoming too alkaline, keeping the hydroxyapatite crystals small and poorly formed.

9.3.3.

Calcium and Phosphate Ions Precipitate and Rupture Secreted Matrix Vesicles

Nucleation of calcium phosphate precipitation within the matrix vesicles is mediated by phosphatidylserine, which comprises about 8% of the phospholipids of the inner cytosolic membrane surface (Fig. 9.5a). Calbindin in the vesicle (Fig. 9.5b) may also contribute. Rapid mineral growth within the vesicle keeps the concentration of dissolved calcium and inorganic phosphate ions so low that additional Ca^{2+} and Pi ions spontaneously enter from the extracellular fluid via their respective transporters. Attached type II and type X collagens from cartilage in the growth plate enhance calcium ion transport and calcification during endochondral ossification (Fig. 9.5b).

Once the solid calcium phosphate reaches a certain size, the vesicle ruptures. The exposed mineral partially redissolves and nucleates the extracellular fluid. Type I collagen fibers propagate crystal growth within the gaps of the quarter-staggered array of fibers and also between the fibers. Within the gaps, serine residues become spontaneously phosphorylated and further nucleate mineralization such that the crystals align parallel to the fibers. The nucleation of collagen fibers is further controlled by alkaline phosphatase removing pyrophosphate, which also accumulates in the osteoid matrix (Fig. 9.5b).

9.3.4.

Structure of the Calcium Transporter Proteins in Matrix Vesicles

The annexins bind to phospholipids in a reversible Ca^{2+} -dependent manner. They are implicated in membrane fusion, vesicular trafficking, and ion-channel formation. X-ray crystal structures of various soluble annexins all reveal a common backbone fold in which each of four repeats in the core domain contains five α -helices connected by short loops (Fig. 9.6a). Two of these loops come together to form Ca^{2+} -binding sites that coordinate with the negatively charged head-group of phosphatidylserine on the cytosolic surface of membranes. Unfortunately, this association does not explain its ion-channel activity. That

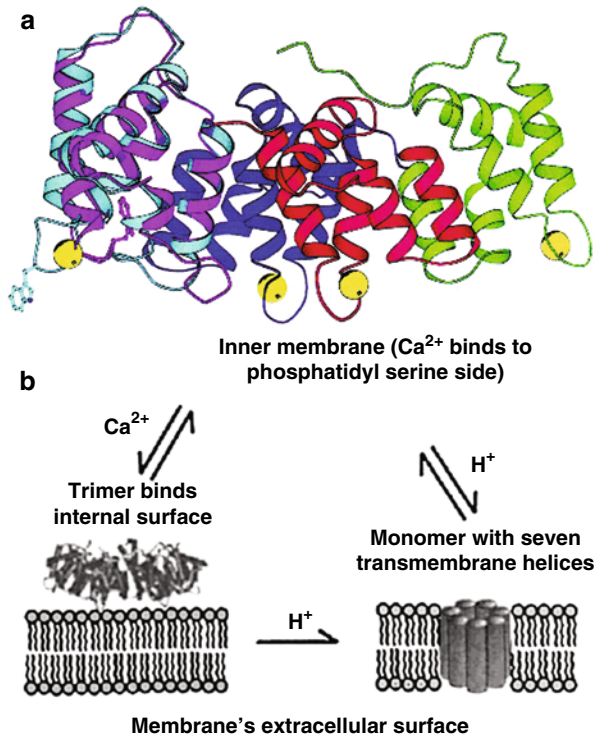


Fig. 9.6 Crystal structure and membrane insertion model of human annexin. **(a)** *Annexin structure.* Annexins all possess four amino acid sequence repeats in the core domain. X-ray crystallography indicates that each repeat has five right-turn helices connected by short loops, two of which come together to form a very tight Ca^{2+} -binding site that coordinates with phosphatidylserine residues on the cytosolic side of a membrane. Different colors highlight the four annexin repeat sequences I through IV: *green, blue, red, and violet* (or *cyan*). High and low Ca^{2+} binding forms are due to a conformational change in repeat III to expose Trp-187 for insertion into the membrane (high binding form is in *cyan*). The bound Ca^{2+} ions are depicted as *yellow spheres* (Image is Fig. 2 from Gerke V and Moss SE (April 2002) “Annexins: from structure to function.” *Physiological Reviews* 82(2):331–371. With permission from the American Physiological Society). **(b)** *Ca^{2+} binding and membrane insertion modifications.* How annexin V inserts into bilayers to create a calcium channel is poorly understood. One possibility is that when annexin interacts with the phosphate residues of the membrane, the acidic environment protonates its four loops, causing a rearrangement into a seven helix transmembrane form that can potentially transport Ca^{2+} . Annexins V and XII are each reversibly converted between three states: surface trimer, phosphatidylserine binding monomer, and transmembrane monomer. Equilibrium is regulated by the concentration of H^+ and Ca^{2+} . It has not yet been determined whether the transmembrane form can be directly converted to the surface trimer when pH and Ca^{2+} concentrations are raised (Modified from Fig. 7 in Isas JM et al. (2000) “Annexins V and XII insert into bilayers at mildly acidic pH and form ion channels.” *Biochemistry* 39(11):3015–3022)

action may be mediated by a form of the protein that inserts into the hydrophobic core of the vesicle lipid bilayer.

Annexins isolated from chondroblast matrix vesicles may be reconstituted with phospholipids to form calcium ion channels in the complete absence of Ca^{2+} ions. Indeed, annexin V has domains that directly bind calcium ions; glutamate and aspartate residues provide the ion binding site (EF-hand domains). Figure 9.6b illustrates putative annexin V channels that mediate an influx of Ca^{2+} ions into artificial bilayers and liposomes (detectable with a calcium-sensitive fluorescent dye). These *in vitro* annexin Ca^{2+} channels, and also the Ca^{2+} influx into matrix vesicles in cell culture and *in vivo*, are blocked by Zn^{2+} ions, or a derivative of 1,4-benzothiazepine (inhibitor K201).

9.3.5.

The Phosphate Transporter Proteins and Pyrophosphate in Matrix Vesicles

The type III sodium-dependent phosphate (Na/Pi) transporters involved in mineralization are members of the inorganic phosphate transporter (PiT) family, which is conserved in all biology. In osteoclasts, a proton (H^+) gradient instead of a sodium (Na^+) gradient transports Pi. These transporters are antiports: the Na^+ or H^+ is transported out as the Pi is transported in (Sects. 2.2.3 and 10.1.4). All these transporters are composed of repeating alpha helices that weave in and out of a membrane with intra- and extracellular turns (Fig. 9.7). A similar kind of structure is proposed for the annexins that mediate calcium ion transport.

A physiologic phosphate concentration is required for bone mineralization. Lowering the concentration prevents mineralization, but raising it does not ensure precipitation because pyrophosphate is present to inhibit precipitation. The concentration of PPI in cartilage and bone is controlled by three enzymes, two on the outer surface of matrix vesicles (Fig. 9.5b). One is tissue-nonspecific alkaline phosphatase (TNAP), which decreases stromal pyrophosphate and the other is NTP-PPI hydrolase (also called plasma cell membrane glycoprotein-1), which increases it. The progressive ankylosis gene product (ANK protein) is expressed by osteoblasts to add to the pyrophosphate of the osteoid matrix from osteoblast cytosol.

Figure 9.8 outlines how matrix vesicles increase and decrease the concentration of pyrophosphate. NTP-PPI hydrolase synthesizes pyrophosphate from stromal fluid nucleotides, mostly ATP ($\text{ATP} \rightarrow \text{AMP} + \text{PPI}$). Many cells secrete ATP into the extracellular fluid and it passes into the blood plasma where it affects a variety of cells independently of its function in intracellular energy metabolism. In mice, a nonfunctional ANK protein or a deletion of NTP-PPI hydrolase decreases the extracellular pyrophosphate concentration and the phenotype exhibits extensive mineralization. Thus, the hydrolysis of pyrophosphate appears to be a major function of alkaline phosphatase (TNAP) after the calcium phosphate precipitate has ruptured the matrix vesicles. Rapid mineralization of collagen and the rest of the osteoid matrix ensue without a need to transport any more Ca^{2+} or Pi to the region.

Mineralization therefore occurs in bone because of the exclusive co-expression in osteoblasts of type I collagen and tissue-nonspecific alkaline phosphatase (TNAP). The abnormal appearance of TNAP in any cell that also produces fibrillar collagen (ectopic TNAP expression) gives rise to pathological (nonbacterial) mineralization, which is outside the scope of this text.

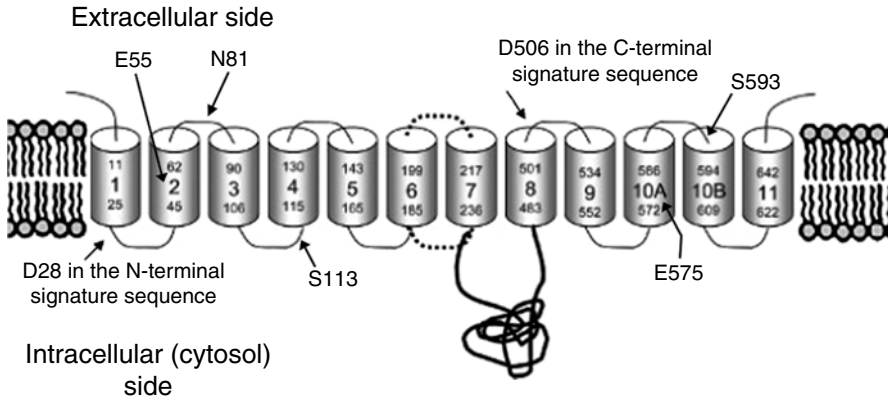


Fig. 9.7 Model of the PiT-2 transporter. There are two homologous transporters in humans, PiT-1 and PiT-2. Diagram illustrates the membrane topology model of PiT-2 derived mostly from transmembrane (TM) predictions, tagging N- and C-terminal sequences, and glycosylated residues. Duplicated sequences are assumed to exhibit similar membrane topologies. The strongest indication for overall orientation of the protein in the membrane is that N81 is glycosylated *in vitro* and therefore extracellular. *Dotted curves* indicate no experimental evidence for the location of the loop between membrane insertions. Amino acids that are important for PiT-2 transport of Pi and Na are indicated with *arrows*: D28 and D506, E55 and E575, S113 and S593. (*Note: Na is in high concentration in all extracellular fluids including the fluid around matrix vesicles and that it spontaneously diffuses through the membrane. In cells, Na is actively extruded by the Na/K exchanger, which is absent from matrix vesicles.*) A consensus amino acid sequence common to all members of this transporter family appears intracellularly before membrane insertion 2, and extracellularly before membrane insertion 9. In addition, all phosphate transporters possess a large cytosolic loop of variable sequences and lengths between membrane insertions 7 and 8 (Image is Fig. 7A from Böttger P and Pedersen L (2005) "Evolutionary and experimental analyses of inorganic phosphate transporter PiT family reveals two related signature sequences harboring highly conserved aspartic acids critical for sodium-dependent phosphate transport function of human PiT2." *FEBS Journal* 272:3060–3074. With copyright permission from Wiley-Blackwell, PO Box 805, 9600 Garsington Road, Oxford, OX4 2DQ, UK)

Osteoblasts secrete osteoid, a matrix rich in type I collagen fibers and vesicles. Precipitation of calcium phosphate is inhibited by a high concentration of pyrophosphate in stromal interstitial fluids, and a high concentration also of albumin and citrate in blood plasma. Pyrophosphate is derived from: (1) transport out of the cytosol, and (2) synthesis from nucleoside triphosphates in the stromal interstitial fluid that permeates the osteoid matrix. Precipitation occurs only when calcium and phosphate ions are taken up into vesicles whose inner membrane is composed of phosphatidylserine. The high concentration of calcium and phosphate ions in the vesicle is mediated by annexin and type III Pi Na-dependent transporters. This overwhelms the pyrophosphate and nucleation occurs. As the precipitate grows and ruptures the membrane, tissue-nonspecific alkaline phosphatase is activated to remove pyrophosphate from the osteoid matrix fluid so that calcium phosphate precipitates around phosphorylated serine residues within the collagen fibers.

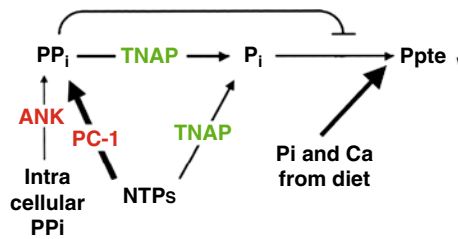


Fig. 9.8 Removal of pyrophosphate is necessary for precipitation. Pyrophosphate (PPi) inhibits the precipitation of calcium phosphate. In the bone matrix, PC-1 (red) is the major producer of PPi from nucleotide triphosphates (NTPs, *thick arrow on left*) and ANK is a minor producer by transporting it from the cytosol of osteoblasts. TNAP (green) causes mineralization by its phosphatase activity converting PPi to two molecules of Pi. TNAP also generates Pi directly from NTPs and PPi, but most Pi and most Ca^{2+} are derived directly from the diet (*thick arrow on right*) (Slightly modified from Fig. 4 in Hessle L et al. (2002) "Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization." Proceedings of the National Academy of Sciences 99:9445–9449. Copyright (2002) National Academy of Sciences, U.S.A)

9.4.1. Non-collagenous Bone Proteins in Bone and Dentin

There are a number of non-collagenous proteins in bone and dentin. Most are proteins of the SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) family. Members of this family are *osteopontin*, *matrix extracellular phosphoglycoprotein* (MEPE), *bone sialoprotein* (BSP), *dentin matrix protein-1* (DMP-1) and *dentin sialophosphoprotein* (DSPP). These proteins are all calcium binding and found mostly in the non-mineralized or mineralized osteoid or dentinal matrix. The SIBLING proteins are encoded together at the same locus on human chromosome 4 and have a similar gene organization in which the downstream encoded exons are the longest and encode an RGD motif that binds integrins (Sect 4.4.1). They also undergo similar post-translational modifications: phosphorylation, glycosylation, proteolytic processing, sulphation, and transglutaminase cross-linking. Extent of expression differs between bone and dentin.

Osteopontin is discussed in Sect. 10.1.1. Little is known about MEPE except that it is required for skeletal development. BSP is hydrolyzed at a single site and DMP-1 at two sites. The fragments bind integrins on the surface of the long processes of osteocytes or odontoblasts, where they likely control apatite growth on collagen so that canaliculi develop in bone or tubules in dentin. DMP1 fragments may also participate in activating osteocalcin expression from osteoblasts (Sect. 9.4.2).

Mice and humans possess a gene that encodes a membrane-bound *gluzincin metalloendopeptidase* (Chapter 8, Table 8.2). This enzyme hydrolyzes small peptides (<3 kDa) on the amino-terminal side of aspartate residues on various proteins. If this gluzincin loses its proteolytic activity due to mutation, BSP and DMP-1 fragments are absent and the osteoid matrix fails to mineralize. There is also an excessive excretion of phosphate into urine (*phosphaturia*) and a low content of phosphate in the blood (*hypophosphatemia*). Rickets

develops as in vitamin D deficiency (Sect. 10.4). The normal (non-mutated) gluzincin prevents phosphate excretion (PHEX) and hypophosphatemia by hydrolyzing an unknown protein in the kidney where BSP and DMP-1 are normally absent. Hypophosphatemia is alternatively caused by a non-functional (mutated) ANK protein or a deleted NTP-PPi hydrolase (Sect. 9.3.5), but then BSP and DMP-1 fragments are present in the osteoid matrix.

Type I collagen is essential for normal bone formation. Improper collagen fiber formation due to mutations in one of the collagen genes, or in the associated processing enzymes, cause skeletal abnormalities called osteogenesis imperfecta (Sect. 7.2.1.), often accompanied by improper dentin development, dentinogenesis imperfecta type I (DGI-I). Other causes of abnormal dentin are due to mutations of DSPP (Table 7.1), dentinogenesis imperfecta types II or III (DGI-II or III), or dentin dysplasia type II (DD-II). In all forms of DGI irrespective of cause, the teeth are discoloured and show structural defects such as bulbous crowns and small pulp chambers. Dentin dysplasia type I (DD-I) is unrelated and characterized by rootless teeth of unknown origin.

One of the first DSPP mutations to be identified was the mutation of a tyrosine residue (encoded as TAT) to an aspartic acid (GAT) at amino acid 6 from the N-terminus. This T → G mutation changes a neutral amino acid to an acidic one that inhibits the binding of the N-terminal secretion signal to the signal recognition particle. The mutated DSPP cannot translocate into the endoplasmic reticulum and is destroyed in the cytosol. Many other mutations of DSPP have since been found, some of which also affect hearing. DSPP appears important for the development of inner ear bones, and it is also expressed by osteoblasts. DSPP mutations are autosomal dominant; a mutation in only one of the two genes results in DGI-II or III, or DD-II. Like other SIBLING proteins, normal DSPP is cleaved. The major products are dentin sialoprotein (DSP, N-terminal end) and dentinophosphoprotein (DPP, C-terminal end). The function of the N-terminal DSP fragment is not known, but the C-terminal DPP fragment is an important initiator and modulator of apatite crystal formation and its content in dentin or bone is exceeded only by osteocalcin (Sect. 9.4.2).

9.4.2. Osteocalcin Is Required for Bone Modeling

The non-collagenous proteins in bone and dentin appear primarily involved in *postmineralization bone modeling*. Osteocalcin is the major noncollagenous protein of bone matrix. It contains 49 amino acids, including three residues of gamma-carboxyglutamic acid (gla), a posttranslational modification of glutamate residues that cause the protein to bind tightly to calcium ions on the hydroxyapatite surface. Osteocalcin, also known as the bone gla protein (BGP), is induced by calcitriol from vitamin D (Sect. 10.4.1). Vitamin A (retinoic acid) is also involved in regulating osteocalcin synthesis. Finally, vitamin K is required to make osteocalcin's gamma-carboxyglutamic acid residues by the same mechanism described for the gla-containing blood-clotting proteins (Sect. 11.2.2). Besides osteocalcin and blood-clotting proteins, some kidney and spleen proteins are also gla-containing.

Warfarin is a poison that inhibits the action of vitamin K by preventing gla residue synthesis (Sect. 11.2.2) and therefore causes an osteocalcin deficiency. Mice in which the

gene for osteocalcin was deleted or given warfarin developed larger bones of improved functional quality. The absence of osteocalcin therefore causes a histologically detectable increase in bone formation without impairing bone resorption. Human studies indicate that osteocalcin in blood plasma is increased in diseases in which bone turnover is high, but decreased in diseases in which bone turnover is low. The association of enhanced osteocalcin production with faster osteoblast turnover, but less bone formation than in osteocalcin deficiency seems contradictory. One explanation is that osteocalcin prevents excessive bone growth due to its gla residues binding tightly to hydroxyapatite and inhibiting unlimited osteoid matrix formation during synthesis.

The osteoblast noncollagenous proteins, bone sialoprotein (BSP), osteopontin (OPN), dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP1), and matrix extracellular phosphoglycoprotein (MEPE) are SIBLING proteins (Small Integrin-Binding Ligand, N-linked Glycoproteins). They bind strongly to Ca^{2+} ions or hydroxyapatite and are involved with osteocalcin (not a sibling protein) in bone remodeling. DSPP is important in dentin formation and mutations cause various forms of abnormal dentin. Two of the SIBLING proteins, BSP and DMP1, are hydrolyzed by a gluzincin (the PHEX protein) in normal osteoid matrix. Absence of the PHEX protein also results in hypophosphatemia, which may prevent skeletal development irrespective of the lack of BSP and DMP1 cleavage in the osteoid matrix. Osteocalcin is the major noncollagenous protein of bone matrix and its gene requires vitamins D and A to activate transcription and its subsequent processing requires vitamin K. A deficiency of osteocalcin appears to enhance skeletal mass, whereas increased levels indicate greater osteoblast turnover. This is useful for categorizing osteoporosis (bone loss) in the elderly. Osteocalcin likely prevents excessive bone mineralization by its gamma-carboxyglutamic acid (gla) residues binding tightly to hydroxyapatite and inhibiting osteoid matrix formation during remodeling. Proper synthesis of bone requires vitamins A, C, D, and K.

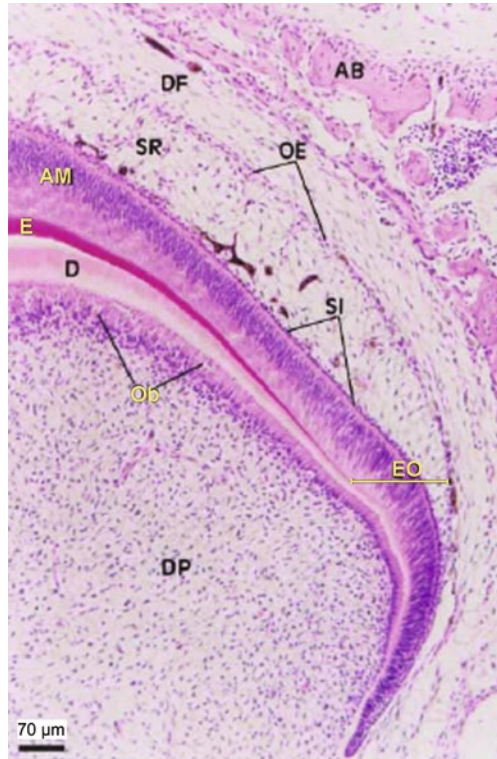
9.5.1.

Enamel Organ and Matrix Development

Teeth develop from tooth buds, an aggregation of cells derived from the ectoderm of the first branchial arch and ectomesenchyme of the neural crest. The tooth bud is divided into enamel organ (EO), dental papilla (DP), and dental follicle. An enamel organ has four layers: outer and inner enamel epithelium, stratum intermedium (SI), and stellate reticulum (SR). The inner enamel epithelium induces the development of odontoblasts from the opposing mesenchymal cells of the dental papilla. As dentin forms, the inner enamel epithelium becomes converted to ameloblasts (Fig. 9.9).

The ameloblasts develop a tall columnar shape in which the nuclei move toward the stratum intermedium. On the dentinal side, a Tome's process appears and enamel matrix is

Fig. 9.9 Light micrograph of a portion of a molar tooth germ from a 3-day-old rat. The enamel organ (EO) shows the outer epithelium (OE), stellate reticulum (SR), stratum intermedium (SI), and the ameloblast layer (AM). A portion of the dental papilla (DP) shows odontoblasts (Ob) adjacent to early dentine (D). *E* enamel, *DF* dental follicle outside the outer epithelium, *AB* alveolar bone. Scale bar = 70 μ m (From Fig. 1 in Cerri PS, de Faria FP, Villa RG, et al. (2004) “Light microscopy and computer three-dimensional reconstruction of the blood capillaries of the enamel organ of rat molar tooth germs.” *Journal of Anatomy* 204: 191–195. With copyright permission from Wiley-Blackwell, PO Box 805, 9600 Garsington Road, Oxford OX4 2DQ, UK)



secreted into a mildly acidic pH that prevents mineralization (*secretory stage*; Fig. 9.10). As the matrix increases in volume, the ameloblast layer (AM) is pushed away from the amelo-dentinal boundary and the Tome's process starts to disappear. A ruffled membrane develops on either side of the resorbing Tome's process and it starts transporting calcium, phosphate, and bicarbonate ions slowly at first and faster as its area increases. The matrix and slow rate of pH increase and mineralization cause carbonated hydroxyapatite crystals to develop and attach end-to-end, forming long enamel ribbons (Fig. 9.10). The direction of the ribbons is determined by the alignment of hydroxyapatite crystals in the adjacent dentinal collagen. As the ribbons mature and mineral ion transport increases, proteases secreted along with the matrix begin to hydrolyze it. This allows the ribbons to thicken and become transformed to rods of hydroxyapatite. The ruffled border functions as an endocytotic membrane and absorbs the matrix protein fragments (*maturation stage*).

Finally, the ameloblasts secrete an unusual basal lamina, which partially mineralizes into the surface enamel crystals, the *enamel cuticle*. By this time, the enamel organ has shrunk so that its outer layers have merged with the ameloblast layer at the completed enamel surface (*postmaturation stage*). This reduced enamel epithelium is rubbed off as the teeth erupt, but the enamel cuticle is abraded more slowly and gradually replaced by proteins from saliva, the *acquired pellicle* (Sect. 12.1.3).

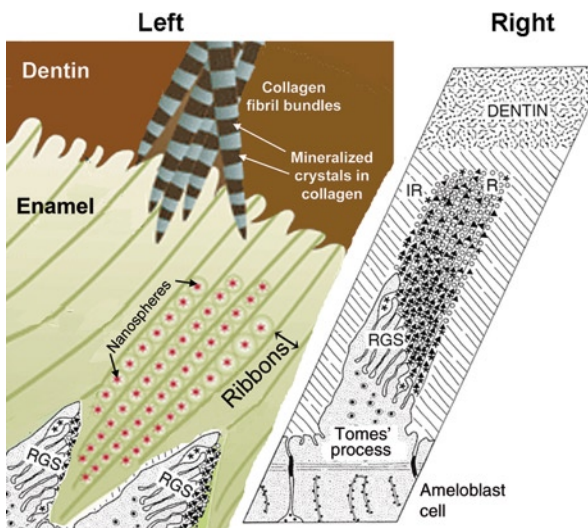


Fig. 9.10 Ameloblasts in developing enamel. This figure is not to scale and oriented upside down with respect to Fig. 9.9; i.e., dentin is above enamel in this figure but below the enamel in Fig. 9.9. *Left:* Amelogenin is secreted from the rod growth site (RGS) of each ameloblast Tomes' process (shown in detail on the *right*). The amelogenin aggregates into nanospheres that bind linearly onto ribbons of carbonated hydroxyapatite. In dentin above the enamel, plate-like apatite crystals grow within collagen fibril gaps (*dark regions center*) before enamel mineralization begins. The long axis of the amelogenin rod hydroxyapatite ("c" axis) parallels the long axis of the collagen fibers that have penetrated between the amelogenin rod ribbons at the enamel–cemental junction (From figure in Veis A (2005) "A window on biomineralization." *Science* (307):1419–1420, March 4, 2005. Reprinted with permission from AAAS). *Right:* Enamel matrix proteins are secreted from Tomes' processes from which the enamel rods extend (rod growth site, RGS). Amelogenin (○) is present in the enamel rods but minor proteins such as intact ameloblastin (*) are more evident than amelogenin (○) within a Tomes' process beneath the rod. In addition, the N-terminal portion (►) of ameloblastin persists into rod enamel, whereas its C-terminal portion is reabsorbed back into the ameloblast. Amelogenin is not detected in the Tomes' process either because its synthesis into the enamel organ (EO) is largely complete before a Tomes' process appears, or it turns over more slowly than ameloblastin. IR inter-rod enamel, R – rod enamel, RGS rod growth site (Reproduced, with permission, from Nanci A, Zalzal S, Lavoie P, et. al. (1998) "Comparative immunochemical analyses of the developmental expression and distribution of ameloblastin and amelogenin in rat incisors." *Journal of Histochemistry and Cytochemistry* 46(8):930)

9.5.2.

Proteins Involved in Enamel Synthesis

Amelogenin is the major protein product of ameloblasts. It is encoded by two highly homologous genes, *Amelx* and *Amely* in the X and Y chromosomes, respectively. The differences in the encoded proteins give rise to minor differences in enamel structure

between males and females. Figure 9.11 gives the amino acid sequence of the amelogenin gene encoded by female mice.

The N-terminal amino acid of secreted amelogenin is number 17 of the encoded protein, as discussed in the legend to Fig. 9.11. The N-terminal domain is hydrophobic, but modified by a phosphorylated serine residue and a hydrophilic tyrosine-rich domain of 12 amino acids. Except for 11 amino acids at the C-terminus (C-terminal domain), the rest of the protein is hydrophobic (Fig. 9.11b). The gene for amelogenin consists of seven exons, of which exon 6 encodes most of the protein as a single, long, hydrophobic domain. Ameloblasts also secrete small amounts of differentially spliced products. In particular, pre-odontoblasts and pre-ameloblasts express an amelogenin variant in which the hydrophobic exon is missing. This short, hydrophilic amelogenin may interact with other proteins or DNA during enamel organ differentiation.

Enamel matrix also contains small amounts of two other proteins, *enamelin* and *ameloblastin*, both hydrophilic. Enamelin is a large protein (1,142 amino acids) that colocalizes with amelogenin on growing enamel crystallites close to the Tomes' process. It is rich in glycine, aspartic acid, and serine, and contains two phosphorylated threonine residues. Most importantly, it contains many N-glycosylated sites from which N-acetylglucosamine residues protrude. These residues noncovalently cross-link the tyrosine-rich region of different amelogenin molecules within the nanospheres (Figs. 9.10 and 9.12). The cross-linking with amelogenin is essential for enamel ribbon development. Ameloblastin (also called sheathlin or amelin; mature form in humans has 421 amino acids) is a cell adhesion molecule located within the Tomes' process (Fig. 9.10, left side) where it likely stabilizes the ameloblasts during matrix secretion. During maturation, its extracellular N-terminal sequence of almost 100 amino acids becomes cleaved off and localizes to the inter-rod space where its functioning is not yet understood.

The enamel matrix also possesses two important proteases: *enamelysin* (MMP20, Sect. 8.3.3) and *enamel matrix serine proteinase* (EMSP1). EMSP1 is closely related to kallekreins, a group of well-characterized serine proteases found in blood plasma and other tissues and known by many different names (ARM1; EMSP; PSTS; KLK4; KLK-L1; PRSS17; MGC116827; MGC116828). The fragments of amelogenin produced by enamelysin and EMSP1 are shown in Fig. 9.11.

9.5.3.

Proposed Mechanism of Enamel Synthesis

Newly secreted amelogenin aggregates into 20-nm nanospheres that promote end-to-end association of carbonated hydroxyapatite crystals into enamel ribbons (Fig. 9.12). Most amelogenin fragments in immature enamel have already been cleaved by enamelysin; all or part of the C-terminus with its adjacent Leucine-Rich Amelogenin Peptide (LRAP) are removed (Fig. 9.11). Only the N-terminal Tyrosine-Rich Amelogenin Peptide (TRAP) segment with its N-terminal LRAP containing a phosphorylated serine residue (residue 16 in Fig. 9.11) remains and holds the amelogenin nanospheres to the carbonated hydroxyapatite (enamel) ribbons. The hydrophobic nanospheres accrete more amelogenin and

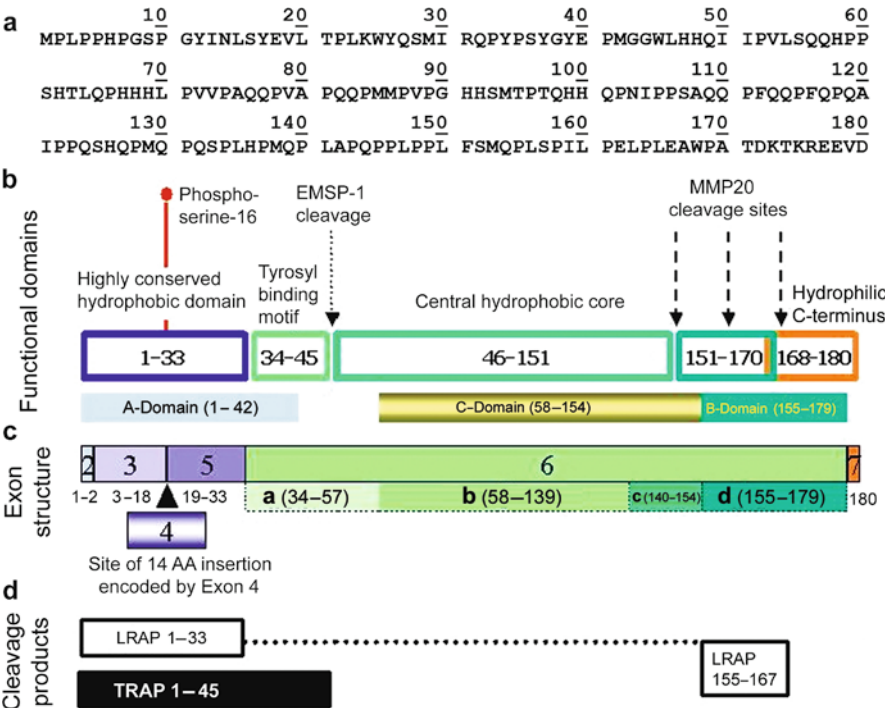


Fig. 9.11 Domains and exon structure of secreted mouse amelogenin. **(a)** *Amino acid sequence.* The sequence is that of secreted mouse amelogenin encoded by isoform 1 of the X-chromosome (amelogenin isoform 1, Accession No. P63277). The 16 amino acid leader sequence, which is excised in the endoplasmic reticulum, is not shown. **(b)** *Functional domains:* The red line and dot beneath the amino acid sequence indicates a single phosphorylation site (serine-16). The major cleavage sites are indicated above the uppermost horizontal bar diagram and the functional domains are indicated beneath. These domains were identified by protein-binding studies. The C-domain contains evolutionarily conserved tandem repeats whose precise function is uncertain. The N-terminal “A” domain includes a tyrosyl-binding motif between amino acids 34 and 45. This domain is cleaved off the remainder of the protein by EMSP-1 (enamel matrix serine proteinase 1) to give the tyrosine-rich amelogenin peptide (TRAP – illustrated in **d**). The C-terminal B domain includes two of three enamelysin (MMP20 metalloproteinase) cleavage sites and the hydrophilic C-terminal domain of 13 amino acids that facilitates hydroxyapatite binding (Fig. 9.10). **(c)** *Exon structure.* The exon structure bar graph lies beneath the domain graph and it illustrates the expressed margins of exons 2 and 7 as well as exons 3, 5, and 6. Exon 1 is not expressed because it lies 5’ of the start-codon. Exon 6 accounts for all but the first N-terminal 33 residues (49 residues if the leader sequence is included) and the amino acid that is the C-terminus. It therefore accounts for the bulk of the secreted protein. **(d)** *Cleavage products.* The common polypeptide fragments, LRAP (leucine-rich amelogenin peptide) and TRAP (see text) are indicated beneath the exon structure graph. C-terminal LRAP peptide (*bottom right*) is cleaved by enamelysin, whereas N-terminal LRAP is cleaved from TRAP (*black rectangle at the foot of the figure*) by EMSP1 (see text). Traces of other proteases then release the N-ter leucine-rich amelogenin peptide, LRAP, and also digest the rest of the amelogenin nanospheres to small peptides, which are endocytosed into the ameloblasts (Modified from a figure titled, “Architecture of the Mouse Amelogenin Gene,” that indicates studies being conducted during 2005/2006 by The Brodie Laboratory at University of Illinois Dental School, Chicago, IL. <http://dentistry.uic.edu/CraniofacialGenetics/ResearchTED.htm>)

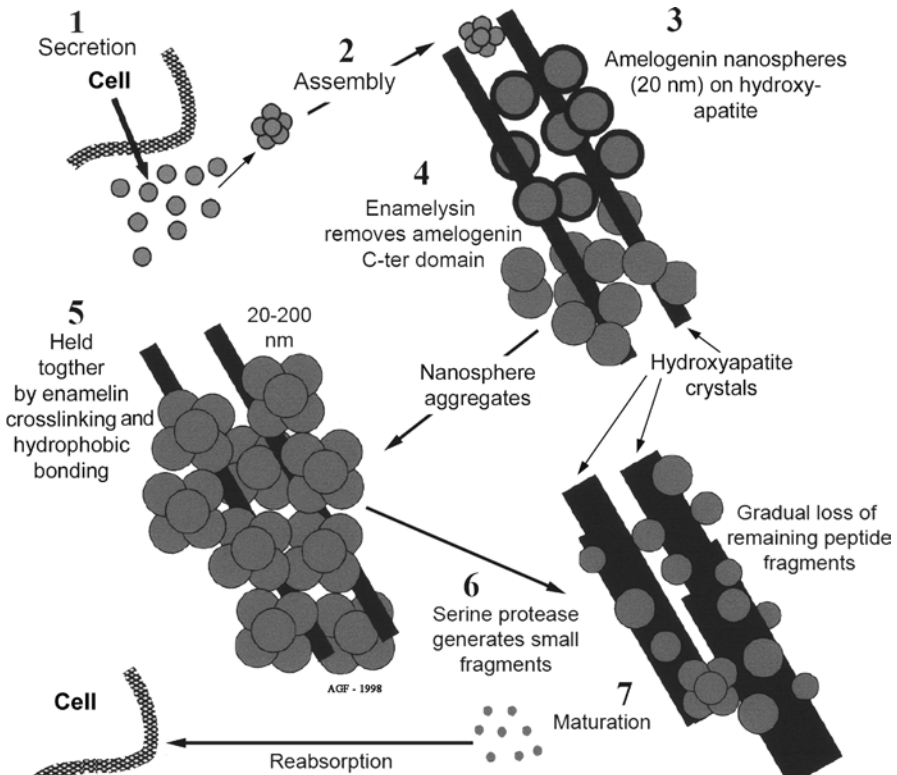


Fig. 9.12 Amelogenin processing in enamel biomineralization. 1 – Amelogenins are secreted as monomers extracellularly. 2 – The monomers assemble to generate nanospheres of 20 nm diameter in which the hydrophilic (anionic) carboxy-terminals are externalized. 3 – The nanospheres interact electrostatically parallel to the *c* axis of small crystals (crystallites) of hydroxyapatite, preventing crystal–crystal fusions and acting as 20-nm spacers that keep the crystallites apart. 4 – Enamelysin processes the exposed amelogenin carboxy-terminal domains, progressively reducing their anionic character. 5 – Hydrophobic nanospheres further assemble by the TRAP region binding enamelin (see text) and this stabilizes the initial enamel crystallites. 6 – EMSP-1 action removes the N-terminal TRAP, which detaches the hydrophobic amelogenin nanospheres. Traces of other proteases generate small peptides that are eventually resorbed by ameloblasts. 7 – As the amelogenin nanospheres thicken and fuse to generate the mature enamel (Reprinted from Fincham AG, Moradian-Oldak J, Simmer JP (1999) “The structural biology of the developing dental enamel matrix.” *Journal of Structural Biology* 126:270–299 with permission from Elsevier). This figure was modified by Dr. Wirsig Weichmann

grow up to tenfold in size restricting crystal extension to the long axis (Fig. 9.10). The TRAP region of amelogenin lies on the surface of the nanospheres and binds tightly but noncovalently to enamelin by the latter’s N-acetylglucosamine residues. EMSP-1 then cuts out the TRAP with its attached enamelin, causing the large nanospheres to contain only the hydrophobic central region of amelogenin and they detach from the enamel ribbon hydroxyapatite crystals. Traces of various other secreted proteases then digest the amelogenin (Fig. 9.12) producing fragments that include the amino-terminal leucine-rich

amelogenin peptide (LRAP) from the N-terminal side of the TRAP (Fig. 9.11). The hydroxyapatite crystals in and around the ribbons develop into rod and inter-rod regions by lateral expansion and fusion. The small peptides circulate and are endocytosed by the ameloblasts.

Mutations that inhibit any of the interactions between the structural proteins (amelogenin, enamelin, or ameloblastin), or that cause a loss of activity of either MMP20 or EMSP1 protease, cause *primary amelogenesis imperfecta*, absence or incomplete formation of enamel without systemic effects. *Secondary amelogenesis imperfecta* is associated with odontogenesis imperfecta due to collagen mutations (Sect. 7.2.1). Abnormal enamel ribbons form due to the altered orientation of the mineralized collagen in dentin. Mutations of laminin-5 and fibrillin-1 and -2 alter stromal organization and may also result in abnormal or absent enamel (Table 7.1).

The inner epithelium of the enamel organ differentiates into ameloblasts after odontoblasts have started to produce dentin. The ameloblasts then: (a) form a Tomes' process through which the protein matrix and proteases are secreted (secretory stage); (b) replace the Tomes' process with a ruffled membrane through which minerals are secreted and peptide fragments of the matrix are reabsorbed (maturation stage); and (c) form an unusual basal lamina that becomes the enamel cuticle (postmaturation stage). Amelogenin has a large, central hydrophobic domain that aggregates into small nanospheres attached to the developing crystals by a hydrophilic C-terminus. Enamelysin removes the C-terminus and the amelogenin forms large nanospheres attached to long ribbons of carbonated hydroxyapatite made from secreted bicarbonate, phosphate, and calcium ions. The TRAP region of amelogenin is on the surface of the nanospheres where it binds to N-acetylglucosamine residues of enamelin. This holds the nanospheres together and to the hydroxyapatite. A second protease, enamel matrix serine proteinase-1 (EMSP1) removes the TRAP with its attached enamelysin, disintegrating the hydrophobic nanospheres. Amelogenin and enamelysin are digested. The crystals thicken into rods that expand and fuse into mature enamel. Genetic mutations of amelogenin and proteases cause primary amelogenesis imperfecta. Mutations of mesenchymal proteins cause secondary amelogenesis imperfecta by causing the enamel ribbons to align incorrectly. Enamel formation requires vitamin A and is independent of vitamins C, D and K. A low blood calcium level has no effect on enamel calcification.

9.6.1.

Summary of Ways in Which Enamel and Bone Differ

The structure of enamel and its mechanisms of formation differ from those of bone, dentin, and cementum in five important ways:

1. Mature enamel is more than 95% mineral (by weight), with only 1–2% matrix protein, and 3–4% water. In contrast, bone, dentin, and cementum are about 70% mineral, 20% collagen, and 10% water. Enamel is brittle compared with bone.

2. Phosphorylation initiates crystallization like serine-phosphorylated collagen, but the mineralization alters enamel matrix structure (nanospheres formation and degradation), unlike the mineralization of collagen matrix.
3. Enamel matrix is partially mineralized before its matrix is completed, whereas bone, dentin, and cementum secrete a preformed organic (osteoid) matrix to which calcium phosphate is added separately from matrix vesicles.
4. Enamel matrix is hydrolyzed to small fragments and disappears after mineralization, whereas the bone matrix remains.
5. Enamel cannot be remodeled, whereas bone, dentin, and cementum may be removed by osteoclasts and replaced with new bone (remodeled; Sect. 10.1.1).

9.6.2.

Summary of the Vitamins for Bone and Enamel Formation

Osteoblasts, odontoblasts, and cementoblasts need vitamin C to make collagen, vitamin D for uptake of calcium into the body (Chap. 10), and vitamins A and K to synthesize and secrete the active form of osteocalcin. Vitamins C and D are the most important during childhood and adolescence: to make adequate amounts of type I collagen and supply a net increase in calcium to the body. Ameloblasts, like other cells of ectodermal origin, require vitamin A to differentiate and secrete their proteins, but none of the other vitamins. Calcification of enamel appears independent of the increase in blood calcium level mediated by vitamin D.

Bones are constantly dissolved by osteoclasts and remineralized by osteoblasts in response to mechanical forces. Osteoclasts possess an acidic compartment and pass demineralized bone products to the periosteum (Sect. 1). They develop in stress-induced bony microcracks and are activated by differentiation factors secreted by osteoblasts, especially after menopause. Menopausal osteoporosis is controlled by drugs that are a stable form of pyrophosphate (bisphosphonate) or cathepsin K inhibitors (Sect. 2). The calcium ion concentration of blood is raised by parathyroid hormone and a vitamin D derivative called calcitriol. Parathyroid hormone causes kidneys to excrete phosphate, retain calcium, and activate calcitriol production (Sect. 3). Calcitriol induces calcium transporter proteins in osteoclasts and intestinal epithelium, where they move calcium from bone or diet into blood (Sect. 4). The chapter concludes with a discussion of calcitonin which lowers blood calcium concentrations by reversing parathyroid hormone effects on the kidney and inhibiting osteoclast activity (Sect. 5).

10.1.1.

Bone Turnover, Osteoclasts, and Lysosomes

Bones are constantly subjected to forces that cause microscopic cracks. These microcracks: (1) attach blood monocytes circulating within the periosteum and bone marrow and (2) induce adjacent osteoblasts to produce cytokines (Sect. 3.3.2) that cause these monocytes to proliferate, fuse, and differentiate into large multinucleated cells called *osteoclasts*. Osteoclasts cause *bone resorption* by acid demineralization and digestion of its proteins by enzymes that are optimally active in an acidic environment. These proteases and other hydrolytic enzymes are stored in a specialized, membrane-sealed compartment (*lysosomes*) into which they are guided by possessing terminal mannose 6-phosphate residues on N-linked glycans.

In osteoclasts, a tunnel or lacuna in the bone develops at a rate of $\sim 50 \mu\text{m/day}$ (Fig. 10.1). The bone dissolution occurs in an *acidic (demineralizing) compartment* that is separated from the rest of the cell by a ruffled membrane whose outer periphery is sealed to the bone

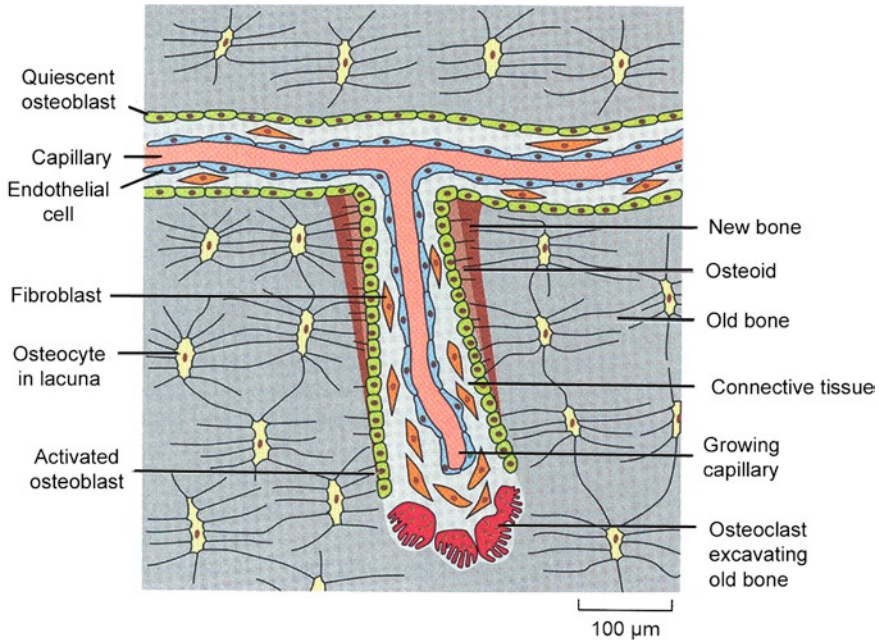


Fig. 10.1 Osteoclasts tunneling. Osteoclasts differentiate at cracks in the bone and form a tunnel (lacuna) containing a central capillary surrounded by connective tissue. Fibroblast-like precursors of osteoblasts are activated to become osteoblasts and new bone is formed (Modified from Fig. 22.54 in *The Molecular Biology of the Cell*. B. Alberts et al., 4th Ed. 2002. Garland Science, Taylor & Francis Group, New York)

by integrin $\alpha_v\beta_3$ (Fig. 10.2). This integrin attaches to an RGD sequence (arg-gly-asg; Sects. 3.2.1 and 8.2.1) on the outer surface of osteopontin, a SIBLING protein that is tightly attached to the outer surface of bone (Sect. 9.4.1.). The ruffled membrane compartment is therefore tightly sealed from the cell and the stroma. Only the small region of bone within the compartment is exposed to HCl, lysosomal proteases, and *acid phosphatase type 5* (Acp5). Acp5 is found in the lysosomes of human kidney, liver, spleen, osteoblast, and osteoclast cells. An iron ion (Fe^{2+}) at the catalytic center is responsible for the enzyme's purple color and also a protein fold known as the metallo-phosphoesterase domain. This domain is present in purple acid phosphatases of plants and in various serine/threonine protein phosphatases. In the bone resorbing compartment of osteoclasts, cathepsin K cleaves out a short central loop from lysosomal Acp5. The modified Acp5 (Acp5b) is optimally active at pH 5.0, whereas the unmodified enzyme in lysosomes (Acp5a) is optimally active at pH 5.5.

The demineralizing compartment contains *acidic, tissue-degrading enzymes* (Sect. 3.3.2) that are secreted from *lysosomes* through the ruffled membrane. Demineralization and proteolysis in this compartment produce calcium ions and peptides that are endocytosed into acidic vesicles. These vesicles traverse the osteoclast ruffled membrane and cytosol and

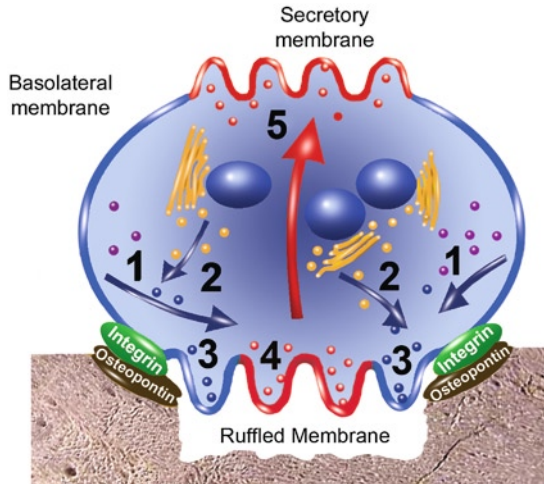


Fig. 10.2 Osteoclast membrane functions. Ruffled and secretory membranes differentiate from the outer osteoclast membrane at opposite ends (see text). Proteases, mostly cathepsin K and MMP-9 are synthesized in the endoplasmic reticulum (ER) (purple dots) and are diverted to lysosomes (blue dots – 1) because the proteases possess N-linked glycans that have attached mannose phosphate residues. Acid phosphatase (Acp5) is also synthesized in the ER and it passes into lysosomal trans-Golgi vesicles (yellow dots) along with the proteases – 2. The vesicles fuse to the cytosolic side of the ruffled membrane (near the integrin/osteopontin seal) and release their contents into the resorption compartment – 3. Protons and chloride ions are separately transported from the cytosol to the bone resorption compartment through this same region (Fig. 10.3). Calcium but not phosphate ions from demineralized bone, along with acid phosphatase and cathepsin K (red dots) enter acidic vesicles in the center of the ruffled membrane – 4. They pass through the cytosol (large red arrow) and are extruded into the extracellular fluid at the secretory membrane – 5 (Adapted from Fig. 1 in HK Väänänen, H Zhao, M Mulari and JM Halleen, “The Cell Biology of Osteoclast Function.” *Journal of Cell Science*. 113:377–381, 2000; Printed in Great Britain © The Company of Biologists Limited 2000 JCS1073; Reproduced with permission of the Company of Biologists ; Figure was modified by Dr. Wirsig-Weichmann)

secrete their contents into the extracellular fluid where it can be picked up by blood capillaries (Fig. 10.2). Simultaneously (Fig. 10.3), transporter proteins move chloride ions from the extracellular fluid to the cytosol and from the cytosol into the demineralizing compartment. Other transporters move phosphate ions in the opposite direction. Protons from carbonic acid in the cytosol are exchanged for released sodium and dihydrogen phosphate. Increased cytosolic acidity likely restricts the osteoclast half-life to ~1.3 days; new osteoclasts must be activated for demineralization to continue. Thus, once all the bone around a microcrack is removed, osteoclasts disappear and osteoblasts lay down new bone in response to the stresses, a process called *remodeling* (Sect. 9.2.1). Cementum and dentin behave similarly, but demineralized enamel cannot be remodeled because new ameloblasts cannot be generated.

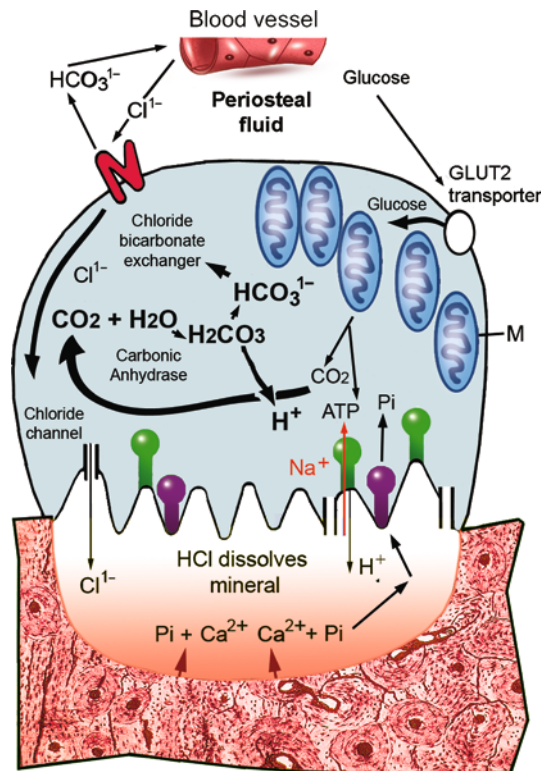


Fig. 10.3 Metabolite and ion exchange in osteoclasts. Glucose in extracellular fluid (*top right*) is transported into osteoclasts by a transporter protein (GLUT2). It is metabolized to pyruvate which enters mitochondria (M – *center right*) to provide ATP, CO_2 , and H_2O . The ATP mediates a transfer of protons (H^+) from carbonic acid (H_2CO_3) through the ruffled membrane in exchange for sodium (Na^+) ions from demineralized bone (*red*). Sodium ions in the cytosol are transferred to the periosteum by the ATP-requiring Na^+/K^+ exchanger common to all cells (not shown). Carbonic anhydrase (CA) makes bicarbonate anions (HCO_3^{1-}) from CO_2 and H_2O in the cytosol. The bicarbonate is exchanged for chloride ions from the extracellular fluid (Cl^{1-} ; *top left*). The chloride ions enter the sealed compartment through channel proteins in the ruffled membrane (*bottom left and right*). HCl accumulates in the demineralizing compartment and dissolves bone, releasing calcium (Ca^{2+}) and dihydrogen phosphate ions and collagen polypeptides that are hydrolyzed by cathepsin K. The phosphate ions buffer the demineralizing compartment so that the pH remains at ~ 4.5 . A proton-dependent phosphate transporter moves sodium dihydrogen phosphate into the cytosol (*purple*) and it passes through the cell to the extracellular fluid by an unknown mechanism. A sodium ion-dependent type III phosphate transporter (Pit-2) in the basolateral membrane (Fig. 10.2) takes up sodium dihydrogen phosphate (Pi) from the extracellular fluid around and transfers it into the cytosol to increase pH. As also illustrated in Fig. 10.2, calcium ions are transported in acidic vesicles to the extracellular fluid (Modified from Figure 2 in Yamashita DS and Dodd RB (2000) “Cathepsin K and the Design of Inhibitors of Cathepsin K.” *Curr. Pharmaceut. Des.* 6:1–24). This figure was modified by Dr. Wirsig-Weichmann

10.1.2. Proteolysis in the Bone Resorbing Compartment

Hydrochloric acid in the demineralizing compartment dissolves the bone mineral and denatures exposed collagen fibers for hydrolysis by cathepsin K. This lysosomal endoprotease (Fig. 10.4a and b) has a cysteine thiol group at its catalytic center (Table 7.1; Fig. 10.4c). Cathepsin K is secreted in larger amounts than other lysosomal proteases and its specificity is due to unique amino acid residues around the peptide binding site. The enzyme hydrolyzes $\alpha_1(\text{I})$ and $\alpha_2(\text{I})$ polypeptides from incompletely denatured fibers in the demineralizing compartment, leaving the cross-linked, telopeptide *ends* (Sect. 4.2.2).

Cathepsin K remains inactive in lysosomes because a propeptide occupies its active site, as in matrilysins (Sect. 8.1.3). Once secreted into the demineralizing compartment along with smaller amounts of other lysosomal proteases, the low pH loosens electrostatic and hydrogen bonds, promoting propeptide cleavage by autocatalysis or other protease action. Mutations that reduce or abolish cathepsin K activity are associated with *pseudohypophosphatasia*, a rare genetic disease characterized in humans by a reduced stature and brittle (osteosclerotic) bones. MMP-9 (neutrophil gelatinase; Sect. 8.3.4) is another proteolytic enzyme in the demineralizing compartment of osteoclasts where it degrades type $\alpha_1(\text{II})$ chains left over from cartilage in endochondral-synthesized bones (Sect. 9.2.2).

10.1.3. Demineralization and remineralization

In addition to collagen peptides and calcium ions, demineralized bones release small amounts of *transforming growth factor-beta*, TGF- β (Sect. 3.2.2). TGF- β is not denatured by acid and not hydrolyzed by lysosomal proteases in the osteoclast demineralizing compartment. Its function is to be transferred to the periosteum during demineralization where it stimulates new bone formation to complete the remodeling process. The TGF- β is endocytosed into acidic vacuoles along with peptides, calcium ions, and Acp5b. The acidic vacuoles pass through the center of the ruffled membrane and are exocytosed into the periosteum or bone marrow through the osteoclast secretory membrane (Fig. 10.2). A greater blood plasma content of Acp5b indicates increased osteoclast activity, but the best indicator of net bone loss is an increased level of type I collagen telopeptides containing pyridinoline (Sect. 4.2.2). A greater amount of telopeptide in blood plasma indicates a greater rate of bone demineralization, whereas a greater amount of osteocalcin indicates a greater rate of bone remineralization. A high telopeptide to osteocalcin ratio in blood plasma therefore indicates net bone demineralization in the body, whereas a low such ratio indicates net bone mineralization.

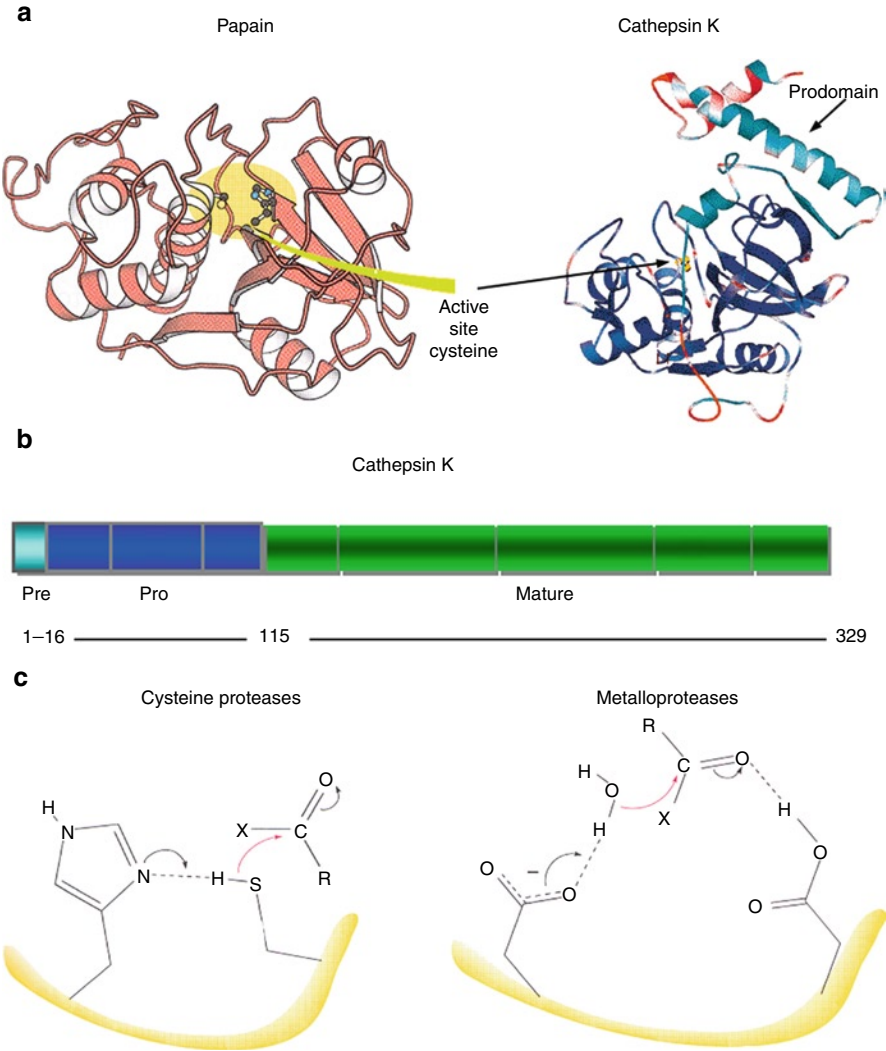


Fig. 10.4 Cathepsin K domain structure and mode of action. **(a)** Ribbon diagrams of papain and cathepsin K structures. Left – papain; right cathepsin K. Similarities of the respective structures and active site regions are obvious (Diagram of papain is part of Fig. 10.18 from Berg., et al., Biochemistry, 5th Ed. 2002, W.H. Freeman & Co., New York; diagram of cathepsin K is Fig. 1 from LaLonde (1999) “The Crystal Structure of Human Procathepsin K.” Biochemistry 38(3): 862–869). **(b)** Domain structure. Signal peptide for protein synthesis into the endoplasmic reticulum (ER) – light blue; propeptide inhibitor region – dark blue; mature protease – green. Gray bands indicate exon breaks. Numbers below indicate the number of amino acids in each domain (Modified from Fig. 1 in Donnarumma, M., et al. (2007) “Molecular analysis and characterization of nine novel CTSK mutations in twelve patients affected by pycnodysostosis. Mutation in brief #961.” Human Mutat. 28(5):524). **(c)** Catalytic mechanism of cysteine (papain family) proteases and metalloproteinases. The major classes of proteases are listed in Table 8.1. They use two fundamentally different catalytic mechanisms to stabilize a tetrahedral intermediate during polypep

10.1.4. Osteoclast Ion and Proton Transport

The ruffled membrane is mostly composed of two *chloride channel proteins*: a *chloride intracellular channel type 5 (ClIC-5)* protein found in the endoplasmic reticulum (ER) and outer plasma membrane; and a *chloride channel type 7 (ClC-7)* protein homologous to voltage-gated chloride channels in the plasma membrane of nerves and muscles (Fig. 10.3). It also contains two other transporters: an ATPase that moves protons (H^+) from the cytosol in exchange for Na ions (Na^+), and a *proton-dependent phosphate transporter* that moves dihydrogen phosphate ions from the bone resorption compartment to the cytosol (Fig. 10.3). The calcium ions are moved out of the bone resorption compartment in acidic vesicles with digested collagen peptides and Acp5b. They are transported through the cytosol and secreted into the periosteum (or bone marrow).

The ATP produced by respiration is required to move the protons from the cytosol to the bone resorption compartment in exchange for sodium ions, fair amounts of which are released from demineralizing bone along with calcium and phosphate. The protons are produced with bicarbonate by *carbonic anhydrase (CA)*, which utilizes the carbon dioxide and water from respiration. The CA catalytic site utilizes a zinc ion, like zincin proteases and alkaline phosphatase. The isozyme in osteoclast is identical to the carbonic anhydrase in red blood cells (CA-II). The sodium and bicarbonate ions in the cytosol are exchanged for extracellular chloride and potassium ions at the periosteal surface. Chloride ions flow from periosteum or bone marrow, through the cytosolic and ruffled membrane chloride transporters to the demineralizing compartment. Sodium ions flow in the opposite direction to the periosteum or bone marrow (Fig. 10.3). Protons and chloride ions accumulate within the bone resorption compartment, causing the pH to fall and the bone to demineralize. Phosphate and sodium ions from the bone are transported out from the ruffled membrane each at the expense of protons moving in and maintaining the demineralizing compartment's acidity.

Despite the substantial amounts of Na^+ ions released from demineralized bone and entering the cytosol in exchange for protons, excessive amounts of dihydrogen phosphate (H_2PO_4) also enter the cytosol. To compensate, there is a separate inward diffusion of disodium monohydrogen phosphate (Na_2HPO_4) from the periosteum/bone marrow through Pit-2 transporters (Sect. 9.3.5) in the osteoclast basolateral membrane (Fig. 10.2). Nevertheless, the pH eventually falls below levels compatible with mitochondrial function, perhaps explaining the osteoclast's short half-life.



Fig. 10.4 (continued) tide bond cleavage. In the cysteine (and also serine and threonine) proteases, the nucleophile is the protease type amino acid (in this case cysteine) which forms a covalent bond with the carbon atom of the bond to be cleaved (covalent catalysis) in contrast to the metalloproteases and aspartic proteases which use an activated water molecule to attack the carbon atom to be cleaved (noncovalent catalysis). In covalent catalysis, a nearby histidine residue normally functions as a base to activate the mechanism, whereas in noncovalent catalysis, the protease type serves as an acid and base, with an ancillary histidine (aspartate proteases) or aspartate or glutamate residue acting as the nucleophile (Fig. 8.2b) (Modified from Fig. 9.18 in Berg., et al., Biochemistry, 5th Ed. 2002, W.H. Freeman & Co., New York)

Stress-induced bony microcracks attract circulating white blood cells (monocytes) that differentiate into osteoclasts possessing an acidic compartment in contact with bone. This extracellular compartment is separated from the cytosol by a ruffled membrane attached by an integrin to osteopontin on the bone surface. Protons are produced by carbonic anhydrase acting on CO_2 and H_2O from respiration and exchanged through an ATP-dependent proton transporter for Na^+ ions from the compartment. Bicarbonate ions in the cytosol are exchanged for periosteal chloride ions that pass on to the acidic compartment through chloride channel proteins in the ruffled membrane. HCl-demineralized collagen fibers are denatured and digested by cathepsin K, an acid-activated, papain-related gelatinase. Calcium ions, collagen peptides, and TGF- β from bone are taken up with cathepsin K-activated acid phosphatase into acid vesicles and exocytosed into the periosteum or bone marrow. Dihydrogen phosphate ions enter from bone through a proton-dependent phosphate transporter and pass through the cytosol separately to the periosteum or bone marrow. Once a microcrack is removed, TGF- β enhances osteoblast differentiation and a stronger layer of bone forms in response to the stress.

10.2.1.

Osteoclast Differentiation

Osteoblasts around bony microcracks are induced to express two cytokines: *monocyte Colony Stimulating Factor* (mCSF) which is secreted and *Osteoclast Differentiating Factor* (ODF) which is mainly on the cell surface. The mCSF stimulates microcrack-adherent monocytes to proliferate and fuse into large multinucleated cells (preosteoclasts) that express *Osteoclast Differentiation and Activation Receptor* (ODAR).

When ODAR attaches to the osteoblast surface-bound ODF, the *receptor/ligand complex activates a membrane-associated tyrosine-protein kinase to induce synthesis of the ruffled membrane*. A tyrosine residue on ClIC-5 (Sect. 10.1.4) is phosphorylated by an activated protein kinase, called *c-src*, the normal *cytosolic* homologue of a viral tyrosine kinase which causes a *sarcoma* (transforms fibroblasts into cancer cells). The phosphorylated ClIC-5 interacts with phospholipids, a chloride channel protein (ClC-7) and two transporter proteins, the ATPase proton transporter, and the proton-dependent phosphate transporter. Mutations that suppress *c-src* or prevent expression or functioning of ClC-7 or ClIC-5 in mice or humans prevent osteoclast development and cause overly dense, brittle bones (osteopetrosis).

Bone resorption is a physiological process prevented by the osteoblast secreting *osteoclast inhibition factor* (OCIF), more commonly called *osteoprotegerin* (OPG). OCIF is a nonmembrane-bound decoy receptor that resembles ODAR and prevents ODF from binding to ODAR and therefore causing preosteoclasts to remain undifferentiated (Fig. 10.5). Bone resorption is related to the body's response to injury or infection, a complex series of events called *inflammation*.

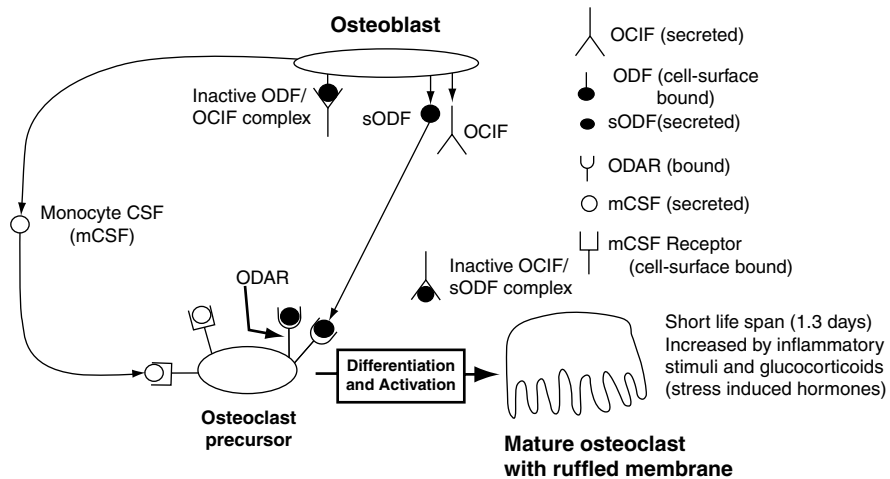


Fig. 10.5 Osteoclast differentiation factors. Osteoblasts make monocyte colony stimulating factor (mCSF) that induces bone adherent monocytes carrying the corresponding receptor (mCSF Receptor) to fuse into osteoclast precursors (preosteoclasts). Preosteoclasts develop within the periosteum and are detectable by their expression of the osteoclast differentiation and activation receptor (ODAR). Osteoblasts also make cell-membrane bound and soluble osteoclast differentiation factors (ODF and sODF) that react with ODAR to cause preosteoclast differentiation. Finally, osteoblasts make osteoclast inhibition factor (OCIF), also called osteoprotegerin, which acts as an ODF decoy receptor and prevents ODF or sODF reacting with ODAR. ODAR is commonly referred to as RANK and ODF as the RANK ligand (RANKL) in the literature (see text)

10.2.2. Osteoclasts and Inflammation

Inflammation is induced by the release of proinflammatory mediators from the cytosol of damaged or infected cells (Sect. 13.2.2). One of the first identified cytokines was an osteoclast activation factor later found to induce a multitude of other proinflammatory events, *InterLeukin-1* (IL-1). A second was *Tumor Necrosis Factor* (TNF), more formally known as tumor necrosis factor- α , TNF- α (Sect. 13.2.2). The binding of these proteins to receptors on adjacent cells activates Nuclear Factor kappa B (NF κ B), a protein in the leukocyte cytosol. The ligand-bound receptor indirectly phosphorylates an NF κ B partner protein (*Inhibitor of NF κ B*, I κ B) in the cytosol. The phosphorylated I κ B is targeted for destruction and its loss exposes a nuclear localization sequence on the NF κ B protein which can now enter the nucleus where it induces the expression of important proteins, depending on the type of cell that is activated. ODAR is a *Receptor Activator of Nuclear Factor kappa B* (RANK), one of a large family of proinflammatory ligand receptors on leukocytes.

When the ODF/ODAR complex activates *c-src* to form a ruffled membrane, it also activates NFκB to activate the transcription factors that induce cathepsin K and acid phosphatase expression. Because ODF binds to ODAR/RANK, it is also known as the RANK ligand (RANKL). OCIF (OPG), the ODF decoy receptor, is RANK without its transmembrane and cytosolic domains. Thus OCIF = OPG and OPG ligand (OPGL) = RANKL = ODF. ODF, ODAR, and OCIF are related to TNFα or its receptor, TNFα family molecules (Table 10.1).

Osteoprotegerin is not restricted to osteoclasts; it is produced by many white blood cells. During lymphocyte development in the central cavity of long bones (Sect. 11.1.1), OPG prevents OPGL binding to RANK on bone-marrow lymphocytes responsible for antibody immunity. Because NFκB is not activated, the lymphocytes proliferate and enter the systemic circulation where they are stimulated by foreign antigens to make antibodies. In the absence of OPG, OPGL binds to RANK and the bone-marrow lymphocytes undergo apoptosis (Sect. 13.4.2). Thus, when bone-marrow lymphocytes recognize a self-antigen, OPG secretion is inhibited and they are eliminated.

Injury, infection, stress-induced hormones (glucocorticoids), parathyroid hormone (Sect. 10.3.1), and increased pressure on a bone all decrease osteoprotegerin (OCIF) production by osteoblasts, causing greater differentiation of osteoclasts, more bone remodeling, and more

Table 10.1 Tumor necrosis factor (TNF)α family molecules in osteoclast development and activation

Type	Acronym	Synonym
<i>Ligand on osteoblast cell surface or secreted</i>	ODF ^a RANKL ^b OPGL TRANCE	Osteoclast differentiation factor RANK ligand Osteoprotegerin ligand TNF-related activation-induced cytokine
	SOFA TNFSF-11	Stromal osteoclast-forming activity TNF superfamily 11
<i>Osteoclast receptor</i>	ODAR ^a	Osteoclast differentiation and activation receptor
	RANK ^b TNFRSF-11A	Receptor activator of NFκB TNF superfamily receptor 11A
<i>Decoy receptor secreted by osteoblast</i>	OCIF ^a	Osteoclastogenesis inhibitory factor
	OPG ^b	Osteoprotegerin
	TR-1	TNF receptor-like molecule 1
	FDCR-1	Follicular dendritic receptor 1
	TNFRSF-11B	TNF superfamily receptor 11B

^aFunctional abbreviation and name used in this book

^bGeneral protein family name and standard name since 2000

(Slightly modified from “Proposed standard nomenclature for new tumor necrosis factor family members involved in the regulation of bone resorption.” Journal of Bone and Mineral Research 15(12):2293–2296, 2000)

Table 10.2 Control of bone metabolism

Causing resorption	Causing calcification
Glucocorticoids (stress hormones)	Growth hormones
Inflammation	Estrogens and androgens
Pressure on bone	Tension on bone
Acidosis (cardiac or lung insufficiency)	

demineralization (Table 10.2). Conversely, growth, anabolic and sex hormones, and tension on the bone, all increase OCIF production by osteoblasts. Few osteoclasts develop and bone resorption is decreased. Cytokines associated with the repair of tissues after injury or infection, specifically IL-4 and IL13, inhibit mechanisms that stimulate osteoclast differentiation and bone resorption. They decrease ODAR synthesis in osteoclasts, and decrease ODF synthesis and increase OCIF synthesis in osteoblasts. Table 10.2 lists the environment, hormones, and inflammatory mediators that influence osteoblast ODF and OCIF (OPG) production and therefore how readily bone resorption can occur.

10.2.3.

Osteoporosis: Major Causes and Therapies

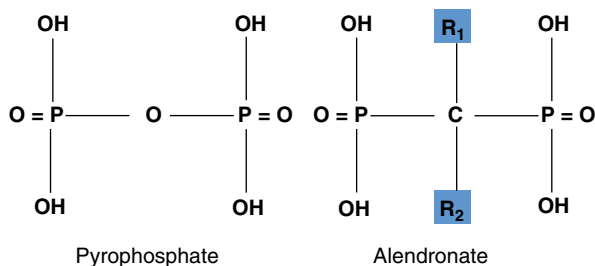
Just as inflammation enhances bone resorption by increasing ODF production locally, the cessation of estrogen production at menopause causes osteoporosis systemically (ODF production increases). Menopausal women therefore suffer a net loss of bone because ODAR (RANK) is activated by ODF (RANKL) in the absence of a compensating increase in OCIF (OPG) production. *The osteoclasts demineralize bone faster than the osteoblasts deposit it.*

Mice overexpressing acid phosphatase (Acp5) develop osteoporosis whereas mice lacking this enzyme have decreased bone resorption and develop mild osteopetrosis (overly dense, brittle bones). Fluoride at 0.12 mM (2.3 ppm) inhibits Acp5b and stimulates osteoblast bone deposition in vitro, but fluoride therapy does not inhibit human osteoporosis (Sect. 16.2.2).

In 1962, pyrophosphate was found to inhibit the spontaneous calcification of urine. Subsequently, the degradation of pyrophosphate to orthophosphate by alkaline phosphatase (TNAP) was found to play a key role in mineralizing collagen fibers during the synthesis of bone (Sect. 9.3.5). Substituting a carbon atom for the central oxygen atom in pyrophosphate (a bisphosphonate) was found to prevent cleavage by alkaline phosphatase.

Because bisphosphonates remain tightly bound to the bone surface and cannot be hydrolyzed, they also retard demineralization. Modifications of the side chains attached to the central carbon atom (Fig. 10.6) has yielded compounds such as alendronate (Fosamax),

Fig. 10.6 Structure of a bisphosphonate showing its relationship to pyrophosphate. Alendronate (Fosamax) is a commonly used bisphosphonate (R_1 is OH and R_2 is $\text{CH}_2\text{--CH}_2\text{--CH}_2\text{--NH}_2$)



which inhibits resorption at doses that minimally block mineralization. Side effects include irritation, inflammation, or ulceration of the esophagus and prevent some patients from using the drug. More recently, the longer and more widespread use of *bisphosphonates* has been linked to *osteonecrosis of the jaw*, especially if used along with some anticancer drugs. Symptoms include jaw pain, numbness, exposed oral bones, loss of teeth, and oral infection. Bisphosphonates are the current drugs of choice to control osteoporosis, but have no effect on pathological bone loss which is mediated by leukocytes (Sect. 13.3.1).

Cathepsin K inhibitors would prevent bone loss without inhibiting mineralization. The compound most tested, Balicatib, forms a covalent bond with the cysteine thiol group at the catalytic center of cathepsin K. Unfortunately, Balicatib tends to concentrate in all lysosomes, so that, over time, amounts in the body will increase and inhibit the catalytic thiol group of other cathepsins, notably cathepsin S which hydrolyzes proteins to peptides for antibody synthesis (antigen presentation). Nevertheless, no adverse side effects were reported in 140 postmenopausal women receiving once-a-day treatment with Balicatib for 12 months, but the possibility of increased infections after some years of taking Balicatib is a potential therapeutic problem.

Microcracks induce osteoblasts to secrete monocyte colony stimulating factor (mCSF) and express cell surface osteoclast differentiating factor (ODF). The mCSF stimulates microcrack adherent monocytes to proliferate and fuse into preosteoclasts expressing osteoclast differentiation and activation receptor (ODAR). The ODAR/ODF receptor/ligand complex: (a) activates a membrane-associated tyrosine-protein kinase to phosphorylate a chloride transporter that assembles the ruffled membrane; and b) activates nuclear transcription factor (NF- κ B) to induce cathepsin K and acid phosphatase. Osteoblasts also secrete osteoclast inhibition factor (OCIF), a nonmembrane-bound decoy receptor that resembles ODAR, but prevents ODF from binding to it. Environment, hormones, and inflammatory mediators influence ODF and OCIF production, and therefore osteoclast-mediated bone resorption. Estrogen inhibits ODF production, so that osteoclasts are activated during menopause, when estrogen production falls. Drugs to treat menopausal osteoporosis include derivatives of pyrophosphate and cathepsin K. Mutations of acid phosphatase cause thicker bones (osteopetrosis). Fluoride inhibits acid phosphatase but not osteoporosis.

10.3.1.

Calcium Metabolism, Parathyroid Hormone, and Calcitriol

Calcium is the most abundant mineral in the human body. About 99% is in the bones and teeth where it plays a structural role and the remaining 1% is in the body tissues and fluids where it is essential for muscle contraction, nerve impulse transmission, and cell metabolism.

Intracellularly, calcium ions are retained in the endoplasmic reticulum, or its specialized form unique to muscle cells, the sarcoplasmic reticulum. In the endoplasmic reticulum, calcium ions are free, chelated to protein, or chelated to phosphatidylserine. Free calcium ions are released through *Transient Receptor Potential* (TRP) calcium ion channels to the cytosol where they bind and stimulate second messenger synthesis. Related channels are involved in transporting calcium ions from the small intestine into the blood plasma, into or out of bone through osteoblasts and osteoclasts, and out of forming urine through the kidney collecting tubule endothelial cells (see Sect. 10.4.1). Calcium ions in the cytosol therefore mediate numerous physiological processes including nerve and muscle activity.

Extracellularly, calcium ions circulate in the blood plasma and interstitial fluid (Sect. 3.3.1). In blood plasma, calcium ions are chelated to albumin and citrate. Albumin (mol. wt. 66,700 kDa) is present at 50–60 mg/mL in plasma, corresponding to 0.9 mmol/L. Although plasma albumin has many different sites that can chelate calcium ions in vitro, only one site binds to calcium ions at physiological albumin concentrations and pH. Thus, albumin binds 0.9 mmol/L of free plasma Ca^{2+} . In addition, citrate (Fig. 10.7), a tricarboxylic acid that the liver secretes into plasma, chelates a free calcium ion to two of its three carboxyl groups, replacing two Na^+ ions. Citrate has a molar concentration of 0.08 mM in venous blood and therefore binds to an equivalent concentration of free calcium. Because the total calcium ion concentration of venous blood is 1.14 mmol/L (range ± 0.2), and the free calcium ion concentration is 0.1 mM, it appears that 0.15 mM of the plasma calcium ion concentration is bound to other plasma components.

The free calcium ions in blood and extracellular fluid are critical for building and maintaining an adequate bone mass, and also for preventing excessive calcification. The sensor that regulates the free calcium ion concentration of plasma is within the parathyroid glands, where it controls the secretion of parathormone (PTH). This 84 amino acid peptide is split from a large, precursor protein and retained in secretory vesicles. If the concentration of free calcium ions drops below a critical level in blood plasma, the gland is activated to secrete PTH into the bloodstream.

Osteoblasts and kidney cells respond to PTH by possessing a single receptor known as the Parathyroid hormone/Parathyroid hormone-Related protein receptor (PPR). The PTH-mediated activation of the osteoblast PPR receptor reduces OCIF (osteoprotegerin)

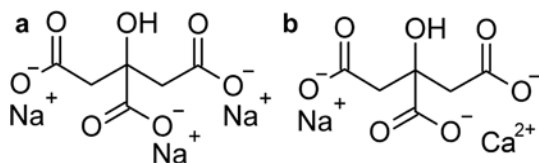


Fig. 10.7 Structure of sodium and calcium citrate. (a) *Sodium citrate*. (b) *Sodium calcium citrate*

Parathyroid hormone-mediated PPR receptor activation of the kidney has three effects (Fig. 10.8). In proximal tubular cells, PTH prevents phosphate reabsorption from the glomerular filtrate (increasing phosphate excretion in urine), and stimulates calcitriol production to activate calcium transport in intestinal epithelial cells, osteoblasts and osteoclasts (Fig. 10.9). The synthesis and mode of action of calcitriol are discussed in Sect. 10.4.1. In distal tubular cells, parathyroid hormone activates Ca^{2+} ion reabsorption from the glomerular filtrate (decreasing calcium excretion in the urine). PTH therefore acts on the kidney to decrease the blood plasma phosphate concentration, and increase both plasma calcium and calcitriol concentrations. The PPR receptor is a Class B *G protein-coupled receptor* (GPCR). An activating ligand such as PTH (an agonist) binds to the GPCR outside the cell and induces changes in the receptor that activate a heterotrimeric G protein (Gs) composed of α -, β - and γ -polypeptide subunits inside the cell. The α -subunit (Gs- α , often written G α s) possesses a non-covalently bound GDP molecule and is tethered by covalently attached fatty acids to the cytoplasmic side of the plasma membrane along with the β - and γ -chains. The changes transmitted across the membrane by the agonist-occupied GPCR cause the Gs- α chain to exchange its bound GDP for GTP in the cytosol. The Gs- α chain containing GTP is *activated* and it spontaneously dissociates from the Gs trimer and stimulates *adenylcyclase* to catalyze the formation of the second messenger molecule, *cyclic AMP* (cAMP). cAMP in turn activates the cAMP-dependent enzyme, *protein kinase A* (PKA). In the kidney, PKA is the effector enzyme that phosphorylates the Pi transporter and stops Pi reabsorption into blood plasma across proximal tubular cells. It also phosphorylates the Ca^{2+} transporter which allows Ca^{2+} reabsorption into blood plasma from distal tubular cells (see also Sect. 10.5.1). In osteoblasts, PKA reduces OCIF secretion and

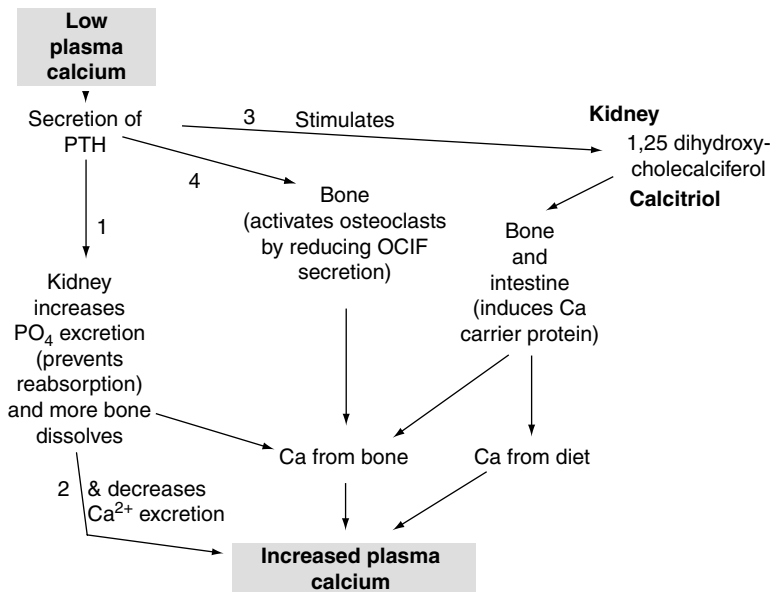


Fig. 10.9 How parathormone raises the free calcium ion concentration of blood plasma. *PTH* parathormone. Effects of PTH are shown by arrows from left to right and down. Effects of calcitriol are shown by arrows running from right to left and down

increases ODF secretion to activate osteoclasts. The Gs- α chain slowly auto-hydrolyses its bound GTP, shutting off adenyl cyclase activation and promoting reformation of the Gs heterotrimer for another round of signaling.

Calcium ions are mostly present in bones or chelated to biological molecules. In blood plasma, only 1% of the calcium ions present are unbound; 78% is bound to albumin, 8% to citrate, and 13% to other plasma proteins. The free calcium ions are prevented from precipitating by plasma pyrophosphate. Calcium ions are also stored in the endoplasmic reticulum (ER), mostly chelated to ER-resident proteins and phosphatidylserine. Free calcium ions may be released through transient receptor potential channels to the cytosol where it activates numerous physiological processes. If the free calcium ion concentration of blood plasma falls, parathyroid hormone (PTH) is secreted by the parathyroid gland cells. PTH speeds up the transport of demineralized bone products by osteoclasts. In the kidney, it increases the excretion of phosphate and decreases the excretion of calcium. PTH also acts on kidney cells to make calcitriol from vitamin D, which induces calcium transporters in the intestine and osteoclasts. PTH mediates these effects by activating G-protein-coupled receptors in the kidney and osteoclasts.

10.4.1.

Vitamin D, Calcitriol, and Calbindins

Vitamin D is a fat soluble vitamin derived from cholesterol. In the human epidermis (skin), sunlight spontaneously oxidizes cholesterol to 7-dehydrocholesterol (Fig. 10.10a). The 7-dehydrocholesterol leaks into the blood where it isomerizes to cholecalciferol (vitamin D₃, Fig. 10.10b and c). Cholecalciferol is enzymatically hydroxylated at C25 in the liver (25-cholecalciferol) and then passes to the kidney where another enzyme is activated by parathyroid hormone to hydroxylate it at C1, forming calcitriol (Fig. 10.10d). The kidney hydroxylase is sensitive to feedback inhibition. As the amount of calcitriol increases, it binds to the hydroxylase and alters the specificity of the kidney enzyme. Additional 25-cholecalciferol is hydroxylated to 24,25-dihydroxycholecalciferol (inactive calcitriol) instead of 1,25 dihydroxycholecalciferol (calcitriol). Other vitamin D derivatives that can be converted to calcitriol are obtained enzymatically from cholesterol in other vertebrates. The most common of these are vitamin D₁ (lamisterol) and D₂ (ergosterol) from cold-water fish such as cod, where their presence keeps membranes fluid at low body temperatures 10–20°C.

Calcitriol behaves like a steroid hormone. It is transported to the nucleus of renal distal tubule cells, intestinal epithelial cells, osteoclasts, and osteoblasts where it induces calbindins, vitamin D-dependent calcium binding proteins. Calbindins mediate the intracellular movement of calcium, from diet to blood, from blood to osteoid matrix, or from bone to blood. There are two calbindin proteins, each encoded by separate genes, one of molecular weight about 9 kDa (calbindin-D_{9k}) and one of molecular weight 28 kDa (calbindin-D_{28k}). Each binds micromolar amounts of calcium and each disappears from animals that are

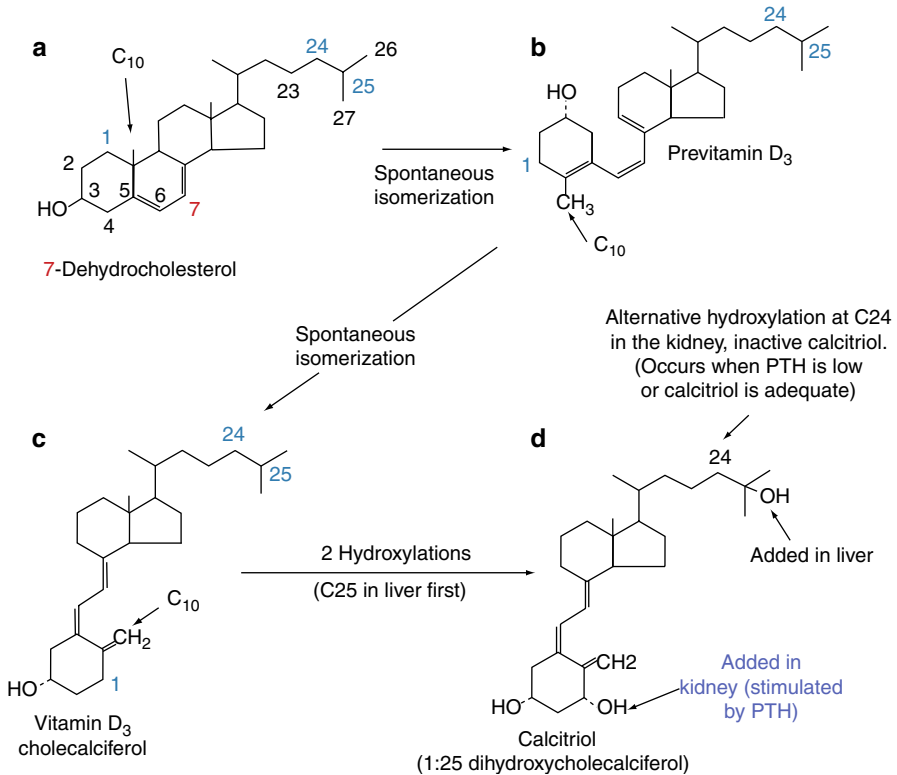


Fig. 10.10 Calcitriol synthesis in the human body. (a) Numbering of the cholesterol carbon atoms at each end of the molecule and position of oxidation site. (b, c) Isomerization to cholecalciferol. (d) Calcitriol showing the hydroxylation site in the liver (*top right*) and kidney (*bottom left*) (Modified from Fig. 27-32 of *Biochemistry*. L. Stryer, 4th Ed. 1995. W.H. Freeman & Co., New York)

deprived of vitamin D. Knockout mice can mediate the necessary calcium transport functions associated with dietary uptake, bone dissolution, and bone formation with calbindin-D_{9k} alone; calbindin-D_{28k} appears important for maintaining osteoblast viability (see legend to Fig. 10.11).

Vitamin D-mediated transcellular calcium transport also involves entry and extrusion. Calcium influx from the intestine or bone is propelled by a steep electrochemical gradient mediated by the superfamily of Transient Receptor Potential (TRP) ion channels noted above. TRP channel ion selectivity and mode of activation are extremely variable. Some are activated or regulated by ligands such as amino acid amines or small peptides, others by physical stimuli (e.g., heat), and still others by as yet unknown mechanisms. All TRP channels are Ca²⁺ and Na⁺ selective, but the selectivity for Ca²⁺ versus Na⁺ (Ca/Na ratio) varies enormously from >100:1, to <0.05:1. This variability has contributed to a confusing TRP channel nomenclature.

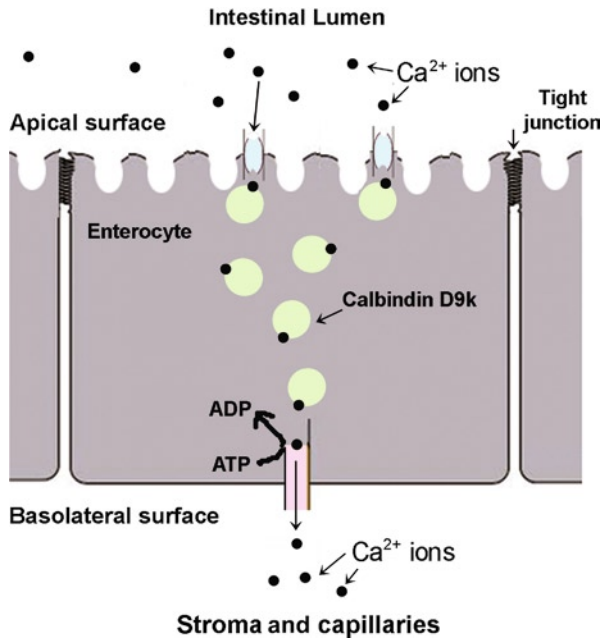


Fig. 10.11 Diagram of vitamin-D dependent calcium transport in intestinal epithelial cells. Dietary calcium ions (filled black circles) in the intestinal lumen enter the epithelial cells (enterocytes) through a Transient Receptor Potential calcium channel family V subfamily 6 transporter protein (TRPV6, pale blue ovals). Tight junctions between the enterocytes (black rectangles on each side of the enterocyte) prevent cell-free diffusion. A related calcium ion transporter (TRPV5) is used in osteoclasts and kidney (see text). Osteoclasts also take up calcium ions using $\text{Na}^+/\text{Ca}^{2+}$ -exchanger-1 (NCX1). Osteoblasts use NCX1 and NCX3 instead of TRPV to take up calcium ions from the blood vessel periosteal stroma. Once inside a cell, Ca^{2+} ions attach to calbindin, mostly calbindin- $\text{D}_{9\text{K}}$, which diffuses through the cytosol to the basal membrane side that contacts the underlying capillary stroma of enterocytes and osteoclasts, or the osteoid matrix of osteoblasts. Calbindin- $\text{D}_{9\text{K}}$ -attached calcium ions are extruded into the stromal matrix of enterocytes, osteoclasts, and osteoblasts by the ATP-dependent Plasma Membrane Ca^{2+} -ATPase 1b (PMCA1b, pink rectangles). Calbindin- $\text{D}_{9\text{K}}$ (pale green circles) predominates in enterocytes and osteoclasts, whereas calbindin- $\text{D}_{28\text{K}}$ predominates in osteoblasts and may protect them and other noncalcium transporting cells expressing calbindin- $\text{D}_{28\text{K}}$ from apoptosis (Sect. 13.4.2). A deficiency of vitamin D prevents cells from expressing either calbindin- $\text{D}_{9\text{K}}$ or calbindin- $\text{D}_{28\text{K}}$ and causes rickets in children or osteomalacia in adults (see text) (Modified from an enterocyte diagram from Wikipedia: http://en.wikipedia.org/wiki/Image:Alpha_Intercalated_Cell_Cartoon.jpg)

In humans, TRP subfamily V member 5 (TRPV5, also called ECAC1 or CaT2) is expressed in osteoclasts and the kidney distal convoluted tubule. TRPV5 is closely related to TRPV6 (ECAC2 or CaT1) expressed in the small intestine (Fig. 10.11) and other tissues. Osteoclasts endocytose Ca^{2+} ions into acidic vesicles from the acidic compartment, pass the vesicles through the ruffled membrane, and exocytose them into the periosteum. However, some Ca^{2+} ions escape into the cytosol where calbindin- $\text{D}_{9\text{K}}$ ferries them to where a $\text{Na}^+/\text{Ca}^{2+}$ exchanger and a Ca^{2+} -dependent ATPase on the membrane extrude it into the stromal extracellular fluid and blood (Fig. 10.11). If the systemic

calcium ion concentration falls, calcitriol is synthesized to activate greater expression of cytosolic calbindin- D_{9K} to speed up Ca^{2+} transport from the acidic compartment. TRPV5 functions in the excretion of this intracellular (nonvesicular transported) calcium to the stromal blood capillaries (of the periosteum). By contrast, osteoblasts use calbindin- D_{28K} to transfer calcium from the periosteal stroma to the osteoid matrix, but as noted above, this function can be subsumed by calbindin- D_{9K} in calbindin- D_{28K} -knockout mice.

10.4.2. Rickets and Osteomalacia

A lack of vitamin D inhibits the transcellular uptake of calcium from the intestine (Fig. 10.11). In growing children, this results in a failure of the osteoid tissue to calcify, causing a skeletal deformity called *rickets*. In rickets, the most obvious symptoms are an outward curvature of the long bones (bowing) of leg and insufficiently mineralized vertebrae that produce a curved spine. In older adults, intestinal TRPV6 expression falls and calcitriol loses its ability to induce calbindins. The low blood calcium levels prevent osteoid matrix from calcifying during bone remodeling; bones incompletely mineralized and fragile. This usually elderly-adult form of rickets is called *osteomalacia* (softened bone). Osteoclast activity is normal and balanced with osteoblast activity, but the osteoid does not mineralize properly. In *osteoporosis* that occurs at menopause (Sect. 10.2.3), osteoclast activity is over-active and osteoblast activity is reduced. A rare form of osteomalacia is caused by hypophosphatemia due to PHEX gluzincin mutations Sect. 9.4.1). Osteomalacia is treated by vitamin D or phosphate supplements, depending on the deficiency.

Vitamin D is a fat soluble vitamin related to cholesterol. In the skin, sunlight spontaneously oxidizes cholesterol to 7-dehydrocholesterol. 7-Dehydrocholesterol spontaneously isomerizes to cholecalciferol (vitamin D3), which is oxidized in the liver to 25-hydroxy cholecalciferol and, under the influence of PTH in the kidney, to 1,25-dihydroxy cholecalciferol (calcitriol), the active form of vitamin D. Vitamin D induces the expression of calcium ion transport proteins (calbindins) in intestinal epithelium, osteoclasts, and osteoblasts. Calbindins and transient receptor potential channels (TRPV) are responsible for the uptake of calcium from the diet. In children, the absence of sunlight provokes a deficiency of vitamin D, causing an absence of calbindins and inadequate blood calcium levels. Osteoid tissue cannot calcify, causing skeletal deformities (rickets). In the elderly, there is a loss of intestinal TRPV receptors and decreased calbindin expression by vitamin D. In both cases, the resultant low blood calcium levels cause poor mineralization during bone remodeling (osteomalacia). Rickets is the childhood expression of osteomalacia. Osteoclast activity is normal but the bone does not properly mineralize. In osteoporosis, the bone is properly mineralized but osteoclasts are overly active.

when blood calcium levels are high. As illustrated in Fig. 10.12, calcitonin injections lower the blood calcium levels back to baseline whereas daily parathyroid hormone injections cause blood Ca^{2+} concentrations to increase.

10.5.2.

Calcitonin and PTH Therapy for Osteoporosis

The direct inhibitory effect of calcitonin on osteoclasts has permitted calcitonin injections to be used to control severe osteoporosis. Surprisingly, PTH can also be used for this purpose. Normally, continuous exposure of bone cells to 0.001–10 nmol/L PTH results in a dose-dependent inhibition of collagen (and bone) synthesis by osteoclasts *in vitro*. The PTH-stimulated osteoblasts reduce OCIF (osteoprotegerin) and increase ODF (RANKL) as already described (Sect. 10.3.1.). Surprisingly, a single daily dose of trace amounts of PTH (0.1–10 nanomol/liter) stimulates OCIF secretion and inhibits ODF action. Because menopause increases ODF secretion (by removing estrogen), intermittent PTH may make OCIF secretion rebound faster.

Daily calcitonin or PTH administration is an effective alternative to bisphosphonates for treating osteoporosis, but much less convenient. Calcitonin and PTH are destroyed by proteases in the intestine and also by proteases in the bloodstream and must be given by daily injection. By contrast, bisphosphonates are not only stable after ingestion, but they also bind to the surface of calcified tissues and are not easily removed. Bisphosphonates can be administered orally, weekly or even monthly. (See Sect. 10.2.3).

A high blood calcium level activates secretion of another polypeptide hormone, calcitonin, from “C” cells of the thyroid gland. Calcitonin acts on G-protein receptors like PRR receptors, but the activation reverses PTH action in the kidney by activating phosphate retention and inhibiting Ca^{2+} ion reabsorption into blood plasma. Although calcitonin inhibits osteoclast activity, its loss (for example, from thyroid carcinoma) does not cause osteoporosis. Conversely, thyroid carcinoma is associated with high levels of calcitonin without osteopetrosis. The calcitonin receptor seems more central to bone development and remodeling. Bone mass appears regulated through hormones that induce the hypothalamus to produce neural mediators that influence RANKL (ODF) production by osteoclasts, in part, by activating nerve fibers present throughout the bone. Intermittent injection of PTH increases osteoblast OCIF secretion and bone deposition suggesting a potential new mechanism for preventing menopausal osteoporosis.

This chapter describes the composition of the vascular system and an overview of how common bleeding problems arise (Sect. 1). A description follows of how blood vessel injury activates platelets and fibrinogen to plug the clot and attract and activate the blood clotting proteins (Sect. 2), how these proteins activate thrombin by extrinsic, intrinsic, and common pathways and how defects in the thrombin activators lead to different forms of hemophilia (Sect. 3). The mechanisms of how a fibrin clot is made and removed for tissue repair (Sect. 4) and prevented from occurring in undamaged (healthy) blood vessels are described (Sect. 5). The chapter concludes with a list of drugs that affect coagulation and of laboratory tests to determine the condition of the clotting system (Sect. 6).

11.1.1

The Vascular System

The vascular system of arteries and veins is directed toward a capillary-mediated flow of interstitial fluid that provides oxygen and nutrients for stromal cells and removes their carbon dioxide and other waste products (Sect. 3.3.1.). This exchange is effective because capillaries are only two cell layers thick, a layer of *endothelial cells* surrounded by a layer of pericytes. The latter cells have some features of muscle cells and they synthesize a reticular fiber matrix which includes actin (a muscle protein), in addition to fibrillin (oxytalan fibers; Sect. 3.1.3), microfibrillar collagen (type VI; Sect. 4.3.4) and delicate collagen fibers (mixture of types I and III; Sect. 4.3.2). The matrix is anchored to type IV collagen of the endothelial cell basal lamina by type VII collagen (Sect. 5.1.1) and held together by proteo-glycosaminoglycans and other adhesive proteins continuous with a loose connective tissue matrix made by nearby stromal fibroblasts (Fig. 11.1). (Sect. 5.1.1). In larger arteries, the region around endothelial cells, basal lamina, and pericytes is called the intima (or tunica intima, Latin for “innermost covering”). Larger blood vessels are surrounded by layers of more differentiated smooth-muscle cells that secrete actin and myosin within an elastic fiber matrix (Sect. 6.2.1). Histologically, the outer stroma is called “tunica media” and “tunica extrema” (middle and outer coverings).

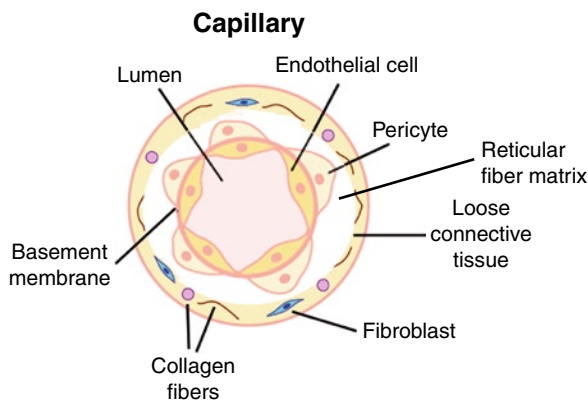


Fig. 11.1 Cross section of blood vessels. Small blood vessels such as capillaries, postcapillary venules and arterioles are lined by a single layer of endothelial cells (ECs), surrounded by a continuous basement membrane (BM). Pericytes (perivascular cells) surround the basement membrane, and are themselves encircled by nearby stromal fibroblasts. The BM underlies the endothelial basal lamina histologically but is biochemically indistinguishable from the reticular fiber matrix synthesized by the pericytes. (Modified from Lafleur MA, Handsley MM and Edwards DR (2003) "Metalloproteinases and their inhibitors in angiogenesis," *Exp. Rev. Mol. Med.* 5(23):1–39, Cambridge University Press: Copyright permission granted by Cambridge University Press, 2008)

The cellular elements of blood, the erythrocytes, leukocytes, and platelets are immersed in blood plasma, a rich solution of proteins, small molecules, and minerals. Most plasma proteins are made and secreted by the liver, but a few are made and secreted by the blood vessel endothelial cells. The cellular elements of blood are made from precursors in the red marrow of long bones (Sect. 9.2.1) by *hematopoiesis*. Aged cells are removed from the circulation by macrophages in the spleen, liver, or bone marrow. Platelets are derived from megakaryocytes, large cells that develop in the bone marrow along with erythrocytes and leukocytes. Platelets are outer membrane-covered secretion granules of megakaryocytes and are removed by macrophages passing through the spleen after only 8–12 days in circulation. *Launching the blood coagulation system requires an interaction between plasma proteins and platelets at a site of injury.*

11.1.2

Bleeding and Blood Clotting Problems

Diseases or drugs that affect the production of platelets or plasma proteins responsible for clotting cause excessive bleeding problems. The most common are diseases of the bone marrow and chemotherapy, both of which deplete platelets by inhibiting

the production of megakaryocytes. In addition, liver disease, commonly caused by chronic alcoholism or viral infections, inhibits the production of the proteins responsible for blood coagulation. Less commonly, genetic mutations of these proteins cause life-long excessive bleeding (*hemophilia*). Finally, although chronic inflammation such as that associated with chronic periodontitis enhances clotting systemically, it often inhibits blood clotting locally (Sects. 13.2.3 and 13.3.1). Any of the above conditions may cause an unexpectedly large amount of bleeding from a tooth extraction and it is advisable to take a medical history and ensure that local inflammation is reduced as much as possible beforehand (Sect.13.5.1).

Capillaries are fragile. Continuous movement of the heart or intermittent movements and pressure changes around the cartilage between the bones stresses the capillaries, predisposing them to wear and tear that can activate the clotting system. Micro-size clots continually form and dissolve. A more severe injury such as bumping into a hard object causes bruising from bleeding beneath the skin. Although bleeding stops after a few minutes because a clot forms, the bruise lasts for a day or longer while leukocytes attracted by proinflammatory cytokines released by the damaged tissues (Sect. 13.3.2) digest the extravascular erythrocytes (Sect. 13.2.4). As the bruise disappears and the damaged tissue begins to be repaired, the clot is gradually lysed.

In older adults, there is a predisposition to clotting because of blood vessel degeneration or a decreased blood flow. The clots may obstruct a small artery or detach as an embolus that obstructs a arteries or veins elsewhere. Possible consequences are heart attack, stroke, pulmonary obstruction, or peripheral necrosis (Sect. 13.4.3). A tooth extraction in an elderly individual can cause an embolism elsewhere in the body. *Elderly patients and patients on medication should be readied for oral surgery only after taking a history to elicit potential bleeding problems and consulting their attending physician.* Rarely, acute disseminated intravascular blood clotting may be induced in any individual by a severe crushing injury, a severe allergic reaction to an insect bite or drug, or uncontrolled systemic bacterial or viral infections.

Capillaries are composed of endothelial cells and an outer pericyte layer that expresses contractile smooth muscle actin within a reticular fiber matrix consisting of thin collagen fibers surrounded by collagen and fibrillin microfibrils. The matrix is held together by proteo-glycosaminoglycans and anchored to the capillary basal lamina. Launching the blood coagulation system requires that platelets be present and activated at an injured site. Most blood clotting proteins are made in the liver; blood cells and platelets are made in the red bone marrow. Diseases of the liver or bone marrow thus enhance bleeding by removing clotting components. Blood vessel degeneration predisposes especially to heart attacks, strokes, pulmonary obstruction, and peripheral necrosis. Disseminated intravascular blood clotting is an often fatal condition induced by a severe crushing injury, allergy or infection.

11.2.1

Blood Vessel Injury, von Willebrand Factor, and Platelets

Platelets initiate coagulation by interacting with von Willebrand Factor (VWF), a multidomain protein (Fig. 11.2) synthesized and secreted by healthy endothelial cells. Following loss of signal peptide in the endothelial cell endoplasmic reticulum (ER), the proVWF molecule dimerizes by disulfide bonding at the C-terminal cysteine knot domain, which is homologous to the domain in TGF- β (Sect. 3.2.2). The dimers are transported to the Golgi, where propeptide domains D1 and D2 are cleaved, permitting large multimers to assemble by disulfide bond exchange between the dimer D3 domains. Sites of stress or injury expose subendothelial stromal collagen that binds to A3 domains in the multimer. The collagen interaction exposes A1 domains that tether platelets from the blood flow by binding to a platelet surface glycoprotein (Gp) called Gplba. Tethering activates the platelets to aggregate into a *platelet plug* or *soft clot* which temporarily stops the bleeding and provides a surface for the more permanent blood clot. Platelet plug formation is enhanced mildly by epinephrine and ADP (Sect. 11.6.6), and strongly if thrombin is already activated (Sect. 11.3.4). Epinephrine is released into the blood as a response to injury, and ADP is released passively from injured endothelial cells and erythrocytes prior to activation. The activated platelets form a plug in three ways: **First**, an integrin on the platelet surface ($\alpha_{IIb}\beta_3$) is activated and binds to an RGD sequence (Sect. 3.2.1) on the VWF C1 domain (Fig. 11.2), but is displaced by *fibrinogen* (1.5 – 3.0 mg/ml in blood plasma, 100 – 300 times greater than multimeric VWF); **Second**, phosphatidylserine is moved from inner to outer leaflet of the platelet membrane; **Third**, *granules* whose contents stimulate *thrombosis* (blood clotting) and wound healing are secreted. One secreted component is *thromboxane* A_2 (TXA₂), an *eicosanoid* (Fig. 13.13.) that stimulates thrombosis. TXA₂ recruits and activates additional

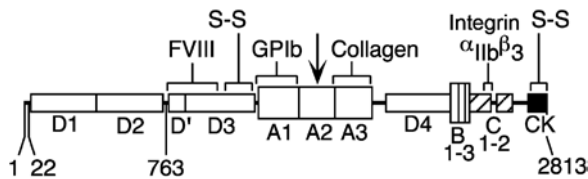


Fig. 11.2 Structure of von Willebrand factor (VWF). The VWF precursor (Expasy accession No. P04275) consists of a signal peptide (residues 1–22) and a propeptide (residues 23–763). The remaining residues 764–2,813 comprise the mature protein, which is divided into A, B, C, D and cysteine knot (CK) domains. The domains are named for sequence homology A1, A2 and A3 are homologous (similar) but unrelated to B, C and D etc. The CK domain is illustrated in detail in Fig. 12.4b. The domains were identified by related repeating sequences in this and many other proteins. Proteins with VWF domains relevant to topics in this book are type VI collagen (legend to Fig. 4.10) and salivary mucin MG1 (Sect. 12.3.1). The VWF binding sites for collagen (A3), platelet glycoprotein Ib, GPIb (A1), integrin $\alpha_{IIb}\beta_3$ (C1) and blood coagulation factor VIII are indicated. The *down-pointing arrow* indicates the site of degradation by ADAMTS-13 between tyr₁₆₀₅ and met₁₆₀₆ (From Sadler JE (2005) “New concepts in von Willebrand disease.” *Annu. Rev. Med.* 56:173–191. Reprinted, with permission, from the *Annual Review of Medicine*, Volume 56 ©2005 by Annual Reviews (www.annualreviews.org))

platelets from blood without VWF binding. Fibrinogen binds to the activated $\alpha_{IIb}\beta_3$ integrin, enlarging the soft clot (plug) at the injured site (Fig. 11.3). The activated platelets further seal capillaries by contracting actin in the pericyte cytosol, and the many platelets in the soft clot provide a large phosphatidylserine surface for coagulation. *Multimeric VWF is therefore the sensor of blood vessel injury; it aggregates and activates platelets to plug the wound and launch the blood coagulation system.*

Multimeric VWF is degraded by an adamalysin, ADAMTS-13, which is secreted into the blood from endothelial cells along with VWF. ADAMTS-13 resembles procollagen N-peptidase (ADAMTS-2; Fig. 8.7b) except that the C-terminal domain of ADAMTS-2 is replaced by four

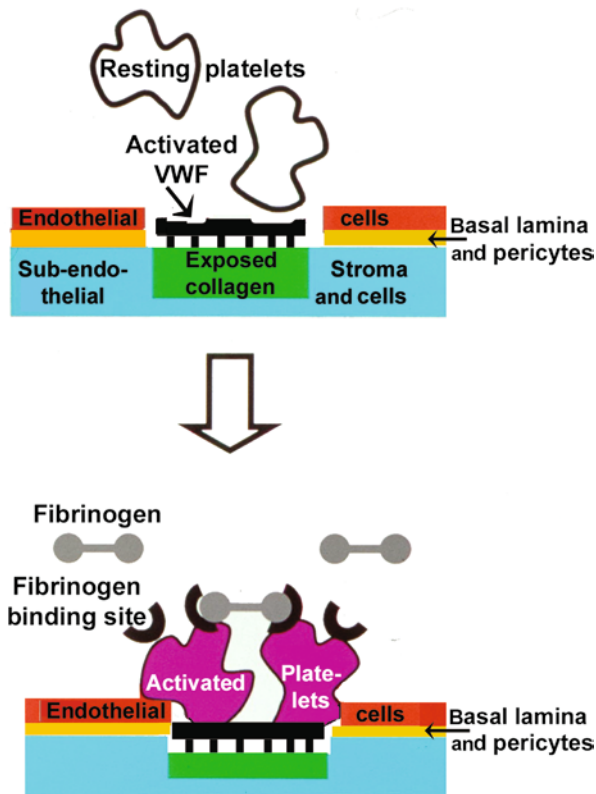


Fig. 11.3 Interactions of platelets with a wounded capillary surface. *Top half:* Resting platelets (white) possess glycoprotein-Ib α . This glycoprotein ligand recognizes a receptor on multimeric von Willebrand Factor (VWF) that becomes attached to exposed collagen (black) after injury has broken the endothelial cell layer and basal lamina (red). *Bottom half:* Upon contact, platelets are activated (maroon) and their surface undergoes two major changes during activation: (i) a fibrinogen receptor (black semicircle) appears and (ii) the phospholipids flip-flop (not shown). The fibrinogen receptor is activated integrin ($\alpha_{IIb}\beta_3$) which binds to a domain on each end of the linear fibrinogen molecule (details of the fibrinogen structure are given in Fig. 11.9). The lipids serve as a surface for localizing and activating clotting factors (Original figure submitted by Dr. Paul DeAngelis, Department of Biochemistry, University of Oklahoma HSC, Oklahoma City, OK, USA)

additional thrombospondin-1 repeats (TS-1 repeats) and terminates in two CUB repeats (like those found on procollagen C-terminal peptidase, PCP, Fig. 8.7a). *Mutations of ADAMTS-13* prevent the degradation of VWF. The concentration of multimeric VWF is increased, oversensitizing the blood to minor injuries at sites such as the heart where capillaries are stressed. The result is *thrombotic thrombocytopenic purpura*, a life-threatening disease in which small clots form, break loose, and block arterioles in the body. In contrast, *mutations of VWF* prevent it from binding collagen and do not stimulate platelets. The blood clotting system is underactivated and injuries cause excessive bleeding (*hemophilia*).

11.2.2

The Gamma-Carboxyglutamate Domain: A Calcium Ion Chelator

Activated platelets possess a phosphatidylserine surface that binds calcium ions (as in matrix vesicles; Sect. 9.3.3). This surface attaches various proteins that circulate in the blood and possess a negatively charged Ca^{2+} binding domain of up to nine γ -carboxyglutamate (gla) residues (Fig. 11.4). Osteocalcin (Sect. 9.4.2.) also possesses this gla domain.

A gla protein is secreted into the endoplasmic reticulum (ER) of the liver where its N-terminal domain binds to and activates an enzyme that converts glutamic acid residues in the gla domain to γ -carboxyglutamic acid. The activated carboxylase enzyme then binds to a reduced form of vitamin K, a *diphenol* (KH_2) to which it adds molecular oxygen. The

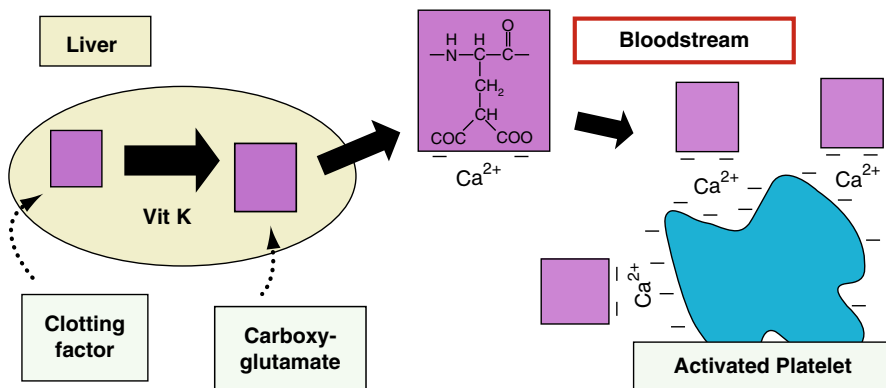


Fig. 11.4 Mechanism of clotting factor localization to an activated platelet surface. *Left:* After synthesis in the liver, certain blood clotting proteins are posttranslationally modified in the endoplasmic reticulum by a vitamin K-dependent Vit K carboxylase. This enzyme forms carboxyglutamate residues (*top center*) that chelate calcium ions. *Right:* In the bloodstream, clotting factor-bound calcium ions attach to negatively charged phosphatidylserine that appears on the surface of activated platelets. Certain therapeutic drugs or acquired deficiencies inhibit this process – see text (Original figure submitted by Dr Paul DeAngelis, Department of Biochemistry, University of Oklahoma HSC, Oklahoma City, OK, USA)

product is an epoxide which reacts with a catalytic amine (N:) on the carboxylase (Fig. 11.5a), converting it to a strong *epoxide base* (K^-) that abstracts a proton from the γ -carbon of a glutamyl residue in the gla domain. The resulting γ -carbanion spontaneously binds to carbon dioxide, converting it to a carboxyglutamate residue (Fig. 11.5b). Simultaneously, the proton (Fig. 5.11a) converts K^- into a stable *quinone epoxide*, KO (Fig. 11.5b). Vitamin K oxidoreductase, a separate enzyme from the carboxylase in the ER membrane, completes the cycle by regenerating KH_2 from KO. The carboxylase

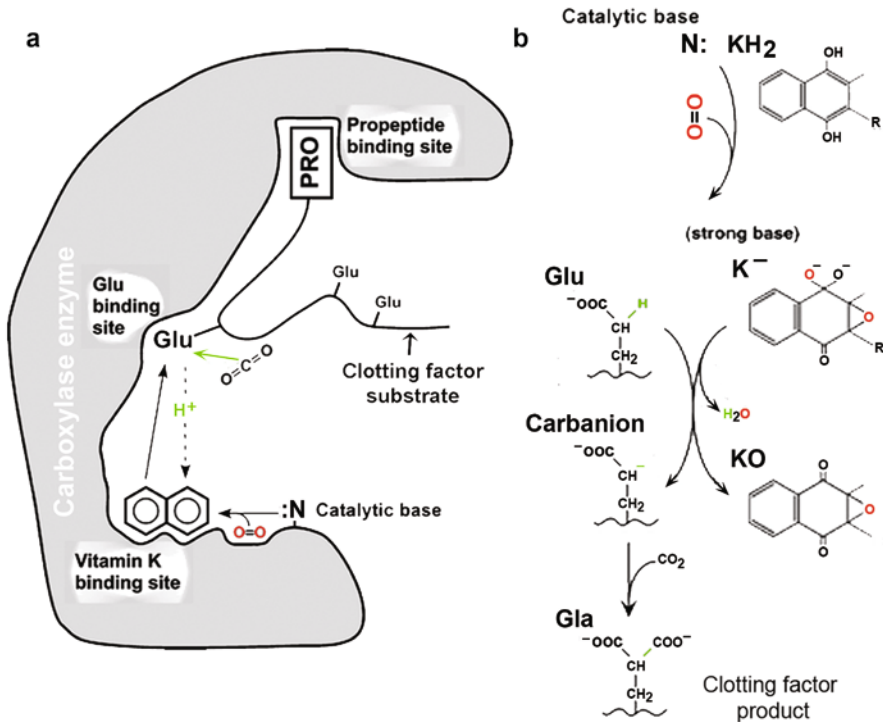


Fig. 11.5 Vitamin K-dependent carboxylase action. (a) *Overall view of the carboxylase.* The vitamin K-dependent protein substrates have high- and low-affinity domains for the carboxylase enzyme. The high affinity site is the *propeptide*, which is cleaved after carboxylation, and the low affinity site is the *most N-terminal glutamate residue to be carboxylated*. Carboxylation is initiated after the second site, the vitamin K binding site, is also occupied by reduced vitamin K (KH_2). A catalytic amine (N:) deprotonates KH_2 , hydroquinone. A strong base epoxide (K^-) forms and attacks a glu residue in the substrate binding domain (*upward pointing solid arrow*). Once this residue is carboxylated, the reactivity of N: is increased (*downward pointing dotted arrow*) and a tightly coordinated complex enables multiple glutamate residues in the substrate to be carboxylated by a processive mechanism (b): *Vitamin K oxidation.* When the strong base epoxide (K^-) generates a glutamyl carbanion that reacts with CO_2 to form gla, the base K^- is oxidized to an intermediate that immediately loses water and forms a quinone epoxide (KO). KO regenerates KH_2 by accepting electrons from glutathione via vitamin K oxidoreductase (Sect. 7.4.1). (From Figs. 1 and 2 in Berkner KL, (2005) "The Vitamin K-Dependent Carboxylase." *Annu. Rev. Nutr.* 25:127–149. Reprinted, with permission, from the *Annual Review of Nutrition*, Volume 25 ©2005 by Annual Reviews (www.annualreviews.org))

Table 11.1 Major coagulation factors by pathway

Extrinsic	Intrinsic	Common	Inhibitory
Tissue factor (TF) (FIII)	FXII ^b	VWF	Protein C ^a
FVII ^a	FXI ^b	Ca ²⁺ ions (FVa)	Protein S ^b
	FIX ^a	FX ^a	
	FVIII	FV (FVa = FVI)	
		Prothrombin (FII) ^a	
		Fibrinogen (FI)	
		Transglutaminase (FXIII)	

^aFactors generating a protease and *with* a gla domain

^bFactor generating a protease and *without* a gla domain

and oxidoreductase iteratively convert all the glutamate residues in a gla-domain. The carboxylase then catalyzes a proteolysis reaction that cleaves the N-terminal propeptide, releasing both carboxylated protein and propeptide separately. Carboxylation of glu to gla resembles oxidation of proline to hydroxyproline by vitamin C during collagen synthesis (Sect. 7.3.1.). The electrons may be obtained from glutathione and passed through an unknown reductase to a cystine disulfide bond in a small hydrophilic loop of vitamin K oxidoreductase protruding into the ER lumen. The electrons are relayed to a second cystine disulfide bond in the membrane of the oxidoreductase before being passed to KO.

Wafarin is one of a family of vitamin K mimics (coumarins) that successfully compete for the KH₂ binding site on vitamin K oxidoreductase. The diphenol cannot be regenerated from KO, and glutamate is not carboxylated. The coumarins are therefore anticoagulants because the gla proteins are secreted into blood as precursors (Coumarins and problems associated with their use are discussed in Sect. 11.6.3) The clotting factor pro-proteases that possess a gla domain are factors II, VII, IX, and X (Table 11.1). These and other factors are carried around in the blood until they touch an activated platelet. The negative charge on the gla domain of the pro-protease completes a calcium sandwich with phosphatidylserine on the platelet aggregate. The negative lipid is on one side, the γ-carboxyglutamate is on the other, and the Ca²⁺ ions are in the middle (Fig. 11.4). Binding alters the conformation of the pro-protease factors and activates proteolysis.

Collagen fibers exposed to blood after an injury are detected by Von Willebrand Factor (VWF), a plasma protein that circulates as multimeric aggregates. On binding collagen, VWF tethers platelets and activates an integrin that binds to an RGD site on the VWF aggregate, but more strongly to fibrinogen. VWF is degraded by an adamalysin, mutations of which enhance clotting. Mutations of VWF enhance bleeding. VWF adherent platelets release thromboxane, activating by-passing platelets to attach fibrinogen by their integrin receptors and a platelet plug or soft clot develops. The platelets in the soft

clot expose a phosphatidylserine surface onto which calcium ions attach blood clotting proteins possessing γ -carboxyglutamate (gla) residues. Processing to gla occurs in the liver where a carboxylase binds to the N-terminal domain of these proteins and converts an internal domain rich in glutamate residues to gla residues. The carboxylase uses a derivative of vitamin K as a cofactor and oxidizes it to a quinone epoxide. The epoxide is reduced by a separate enzyme, vitamin K oxidoreductase, in tandem with gla domain carboxylation. After all of the domain's glutamate residues are carboxylated, the carboxylase cleaves the substrate's N-terminal propeptide, releasing the carboxylated protein and the propeptide separately. The oxidoreductase is inhibited by vitamin K analogues (coumarins) which cause the precursor proteins to be released without carboxylation and unable to bind to calcium ions on a phosphatidylserine surface. Large concentrations of vitamin K reverse the coumarin inhibition by providing large amounts of the initial derivative and allowing the oxidized quinone epoxide to be excreted instead of reduced.

11.3.1

The Extrinsic, Intrinsic, and Common Coagulation Pathways

Following the recruitment and activation of platelets by injured capillaries, their soft clot aggregates provide a surface for plasma proteins to interact and activate thrombin, which transforms soluble fibrinogen to a fibrin clot. Thrombin is activated by two blood coagulation pathways, extrinsic and intrinsic. A defective extrinsic path is incompatible with life, whereas defects of the intrinsic path cause hemophilia.

11.3.2

The Extrinsic Pathway

The extrinsic pathway is mediated by tissue factor (TF), also known as thromboplastin or factor III (Fig. 11.6a). Tissue factor is not present in blood plasma, i.e., it is extrinsic or outside of the vascular system. TF is a membrane protein that becomes exposed to the blood by trauma to the endothelial surface of blood vessels. It is especially rich in the membranes of pericytes, platelets, and leukocytes, but present in lesser amounts in most cells. It is absent from striated muscle cells and chondroblasts.

When TF is exposed to blood, it spontaneously binds factor VII, a serine protease of the coagulation system that possesses a gla domain (Table 11.1). The TF–VII complex therefore adheres to the negatively charged surface of activated platelets. About 0.4% of factor VII is activated in blood by fatty acid- or triglyceride-mediated activation of factor IX and binds similarly to TF. The TF–VIIa complex (Fig. 11.6a) has proteolytic activity and cleaves (converts) another gla protein that also binds to the activated platelets, factor X. Factor Xa is a serine protease that converts the remainder of the TF–VII complex, generating additional Xa proteolytic activity that ultimately converts prothrombin to thrombin for

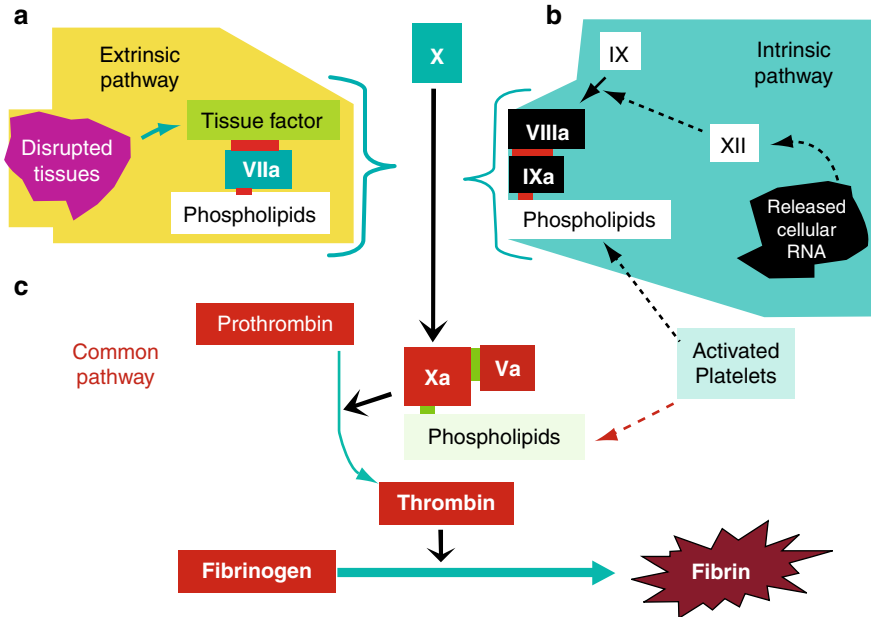


Fig. 11.6 Extrinsic and intrinsic pathways. **(a)** *Extrinsic pathway* (top left): When an injury activates platelets to form a phospholipid surface (white rectangle, lower right), disrupted tissues (magenta, top left) release tissue factor (TF) (green). Factor VII and its activated derivative (factor VIIa, blue) in blood plasma bind to the phospholipid surface by their gla domain and attach tissue factor. Unconverted factor VII is activated by small amounts of VIIa present and bound to TF. Factor X (dark blue) binds similarly to phospholipid and is converted by the TF-VIIa complex to Xa (red). **(b)** *Intrinsic pathway* (top right): Injury releases RNA, a negatively charged polymer that converts factor XII to XIIa (white squares). Factor XIIa activates factor XI (not shown), which remains soluble and activates factor IX bound to phospholipids (black square). Factor IXa activates bound factor X so that it weakly converts prothrombin to thrombin. The thrombin then activates factor VIII to VIIIa (black rectangle), which binds to factor IXa and accelerates its conversion of phospholipid-bound factor X. **(c)** *Common Pathway* (bottom center): Increasing amounts of thrombin activate factor V, converting it to Va. Va (small red square) binds to factor Xa (large red square) on the phospholipid surface and greatly accelerates the conversion of prothrombin to thrombin (red rectangles), and of fibrinogen (red rectangle) to fibrin (brown star). **Factor numbering** describes discovery, not interaction. More abundant factors were discovered and numbered first, fibrinogen (factor I), thrombin and prothrombin (factors IIa and II). Today, these factors are usually referred to by their names for clarity. The later-discovered factors are still referred to by their numbers. Factor XII was the last factor to be discovered because it is first in the cascade and present in least amounts in blood. A few molecules of factor XII activate more molecules of factor XI and factor IX, causing the intrinsic path to activate factor Xa rapidly (**a**, **b**, and **c** are original figures submitted by Dr. Paul DeAngelis, Department of Biochemistry, University of Oklahoma HSC, Oklahoma City, OK, USA)

fibrin generation at the injured site. *TF primes the coagulation system just as VWF primes platelet activation.*

Excessive production of factor VIIa is associated with an unusual susceptibility to thrombosis in heart blood vessel. Unlike striated muscles, smooth muscles such as the heart possess TF. The amount of factor VIIa in blood may be increased by genetic promoter mutations, but more commonly by increased blood triglyceride levels. The conversion of circulating factor IX to factor IXa in the blood of obese or diabetic individuals results in an increased activation of factor VII. Because shear forces in the heart continually damage arterioles and capillaries, individuals with more factor VIIa have an overly primed extrinsic coagulation path, making them susceptible to thrombosis (heart attack). The capillaries are similarly injured around the joints, but these regions are protected from clotting because, as noted above, joints have no tissue factor.

11.3.3

The Intrinsic Pathway

The intrinsic pathway is activated by blood coming into contact with a negatively charged surface. In vitro, glass is negatively charged, and blood in contact with a glass tube or glass slide readily coagulates. The intrinsic pathway is illustrated in Fig. 11.6b. The key initiator is factor XII (Hageman factor), a weak serine protease that binds to all negatively charged surfaces.

In vivo, Hageman factor appears activated by small amounts of RNA from crushed cells. Once bound to a negative polymer, Hageman factor changes conformation so as to greatly increase its protease activity ($\times 10^5$). Nevertheless, there is so little of factor XII that factor XIIa has little absolute activity – only enough to cleave factor XI (listed in Table 11.1, but not shown in Fig. 11.6b). Factor XI is also present in small amounts in blood, and its activated factor remains in solution. Enough of factor XIa is produced to cleave factor IX that has already bound by its gla domain to phosphatidylserine on activated platelets. Factor X similarly binds to the platelet aggregate. Factor IXa weakly activates factor X, which, in turn, produces small amounts of thrombin from prothrombin. The traces of thrombin activate factor VIII, a nonproteolytic cofactor accelerator that binds to factor IXa, increasing its ability to convert factor X to Xa.

11.3.4

The Common Pathway

Irrespective of whether factor X is activated by extrinsic or intrinsic pathways, it catalyzes the activation of thrombin from prothrombin (Fig. 11.6c). Initially, activated factor X (factor Xa) produces small amounts of thrombin that cleave factor V, which is more sensitive to thrombin than even fibrinogen, thrombin's major substrate. Factor Va is a *non-proteolytic cofactor accelerator*. It binds to factor Xa and greatly increases the

rate of Xa catalyzed conversion of prothrombin to thrombin. Factor Va therefore resembles factor VIIIa, which accelerates the production of factor IXa in the intrinsic path. The production of factor Va is therefore the key activation step of the common pathway, and it generates large amounts of thrombin from prothrombin (Fig. 11.6c).

Thrombin mediates blood clotting by catalyzing fibrinogen to form the fibrin blood clot. Activation by factor Xa involves two cuts of prothrombin. The first cut removes the γ -carboxyglutamate domain and allows binding to glycoprotein-Iba of platelets trapped in the soft clot. This binding positions the carboxyglutamate-free prothrombin beside factor Xa, which completes the conversion to thrombin by cutting the polypeptide into two. The two clipped segments remain attached by disulfide bonds (Fig. 11.7), permitting a reorientation of the polypeptide fragments that activates thrombin's serine protease activity. This protease also cleaves factor XI to factor XIa and enhances intrinsic path activation. Once thrombin has catalyzed the cleavage of fibrinogen, it remains fibrin-attached where it also catalyzes the activation of factor XIII, a transglutaminase cross-linking enzyme in plasma that strengthens the fibrin clot.

Thrombin has two binding sites for exogenous partners or ligands called exosites (Fig. 11.8). Exosite I binds to fibrinogen, converts it to fibrin and remains bound to the fibrin. Conversion of fibrinogen to fibrin is enhanced by thrombin also binding to platelet GpIba glycan at exosite II. The thrombin-platelet complex also activates a plug from

Fig. 11.7 Cleavage of prothrombin by factor Xa. The γ -carboxyglutamate (gla) domain attaches prothrombin to the activated platelets. The first cleavage of the prothrombin polypeptide by factor Xa removes the γ -carboxyglutamate domain and the second activates the protease as the thrombin is released from the membrane (Original figure submitted by Dr. Paul DeAngelis, Department of Biochemistry, University of Oklahoma HSC, Oklahoma City, OK, USA)

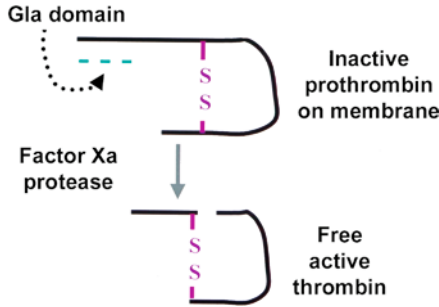
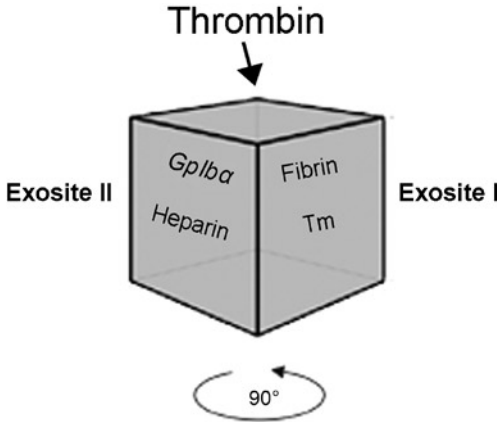


Fig. 11.8 Thrombin–cofactor interactions. In the activated thrombin molecule, exosite I lies at right angles to exosite II. Exosite I binds either fibrin or thrombomodulin (TM) depending on what glycan binds to exosite II, glycoprotein-Iba (GpIba) or heparin. See Sects. 11.3.4 and 11.5.1



fresh platelets in the blood (Sect. 11.2.1). Otherwise it diffuses into the tissues where exosite II instead binds to glycosaminoglycans that alter exosite I conformation and functions. The functions of ligands other than fibrinogen/fibrin and GpIb α are discussed in Sect. 11.5.2. Fibrin formation is discussed in Sect. 11.4.1.

11.3.5 The Hemophiliac (Excessive Bleeding Diseases)

The most common form of hemophilia is due to Von Willebrand disease in which VWF is mutated at the site where it binds to collagen (A3 domain) or to platelets (A1 or C1 domain). The second most common form is classical hemophilia (hemophilia A), long present in European royal families. Much of our original understanding of blood coagulation relied on identifying and studying these two forms of hemophilia.

In hemophilia A, the protein sequence of factor VIII is mutated so that it cannot be cleaved, resulting in a blocked intrinsic pathway. Therefore, tissue factor must be activated to stop bleeding (extrinsic pathway). Because the capillaries of joints and muscles are continually damaged by crushing when the surrounding bones and muscles move, they produce small amounts of RNA that activates the intrinsic path. Because there is no tissue factor at that site and the mutation of factor VIII in hemophilia A has blocked the intrinsic path, the affected subjects bleed into the joints. The pressure in the joint eventually stops the bleeding, and reticuloendothelial cells are recruited to remove the blood cells. The pain is relieved, but the joint structure is slowly destroyed and over time surviving individuals develop arthritis.

Hemophilia B is rarer than hemophilia A and is sometimes called Christmas disease after Stephen Christmas, the first patient described with this disease. It is due to a mutation that prevents factor IX from activating factor VIII. However, unlike hemophilia A, factor VIII can be activated by thrombin, and so hemophilia B is less severe.

Hemophilia C occurs at 10% of the frequency of hemophilia A and is the least common form of hemophilia in the United States, where it mainly occurs in Ashkenazi Jews. It is caused by a deficiency of factor XI. Unlike hemophilia A and B, there is no bleeding into joints; Factor XIIa can apparently activate factor IX directly without first activating factor XI. Nevertheless, afflicted individuals suffer nosebleeds and heavy menstrual bleeding and, like all hemophiliacs, require a clotting agent to prevent excessive bleeding following a tooth extraction (Sect. 11.6.5).

Two pathways initiate a fibrin clot. *Extrinsic path* is mediated by tissue factor, also called thromboplastin. This membrane protein is exposed when pericytes are damaged. It binds to factor VIIa in blood. Factor VIIa is a protease and the phospholipid–VIIa–TF complex activates (converts) factor X by cleaving it to Xa. *Intrinsic path* is initiated by factor XII (Hageman factor), whose conformation is changed to a protease (XIIa) by contact with a negatively charged surface such as RNA from damaged or necrotic cells.

Factor XIIa activates factor XI, which, in turn, activates factors IX and X attached to platelets aggregated by tissue damage. Xa cleaves prothrombin and makes enough thrombin to activate factor VIII (IXa accelerator). The phospholipid–IXa–VIIIa complex activates factor X. *Common path* is mediated by Xa activating factor V (Xa accelerator). The platelet membrane bound Xa–V complex is a strong activator of prothrombin and activates enough thrombin to make fibrin from fibrinogen (clot). There are four classes of *hemophilia* (inadequate thrombin production): (1) *Von Willebrand disease* – mutations that inhibit VWF binding to collagen or platelets; (2) *Hemophilia A* – mutations of factor VIII that inhibit IXa accelerator activation; bleeding occurs mainly because of intrinsic path deficiency in joints; (3) *Hemophilia B* – mutations of factor IX conversion of factor VIII, but factor Xa conversion is unaffected; side effects less than those of Hemophilia A; (4) *Hemophilia C* – deficiency of factor XI; side effects even milder than Hemophilia B because XIIa activates some factor X directly.

11.4.1

The Fibrin Blood Clot: Production and Prevention

Blood clots are composed of fibrinogen, an abundant negatively charged protein in blood plasma Sect. 11.2.1. Fibrinogen is also secreted from the granules of activated platelets. It is composed of three interdigitated, parallel polypeptides, $\text{A}\alpha$, $\text{B}\beta$, and γ , that are disulfide-bonded. Each trimer is dimerized near its N-terminus by an additional disulfide bond between two $\text{A}\alpha$ chains (Fig. 11.9a). The hexamer forms a dense central node (E node, orange) and two on either side (D nodes, blue; Fig. 11.9b). The E node consists mainly of globular segments of the $\text{A}\alpha$ and $\text{B}\beta$ polypeptides, whereas the two D nodes are each composed of the $\text{B}\beta$, and γ chains. The $\alpha_{\text{IIb}}\beta_3$ integrin of activated platelets attaches to a single site on a D node to aggregate them (Fig. 11.3).

Polymerization results from the proteolytic activity of thrombin specifically removing fibrinopeptides A and B from the amino-terminal ends of the $\text{A}\alpha$ and $\text{B}\beta$ peptides (Fig. 11.9b–c). This cleavage is N-terminal to the E node and exposes four knobs (Fig. 11.9c), each of which fits into a pit present in the D node of fibrinogen or fibrin. The reaction initially creates a fibrin dimer that polymerizes laterally (Fig. 11.9d–e). The polymer becomes crosslinked by simultaneous, thrombin-induced conversion of factor XIII to transglutaminase (factor XIIIa) that covalently attaches the glutamine amide residues to spatially adjacent lysine residues (Fig. 11.9f) as occurs in fibrillin (Fig. 6.3). At first, up to six such crosslinks form between γ chains on the surface of the D-node. Additional crosslinks form later between α chains (Fig. 11.9f) that lie alongside the D node (Fig. 11.9f). Fibronectin, which is secreted by activated platelets along with fibrinogen, becomes cross-linked into fibrin, making the clot stronger and more resistant to degradation than fresh fibrin. The fibrin shrinks and a clear fluid called serum (*blood plasma from which fibrinogen was removed by conversion to fibrin*) is released. This fluid is the major component of inflammatory exudates where fibrin clot formation is prevented by excessive plasmin activation (see next section and Sect. 13.1.2).

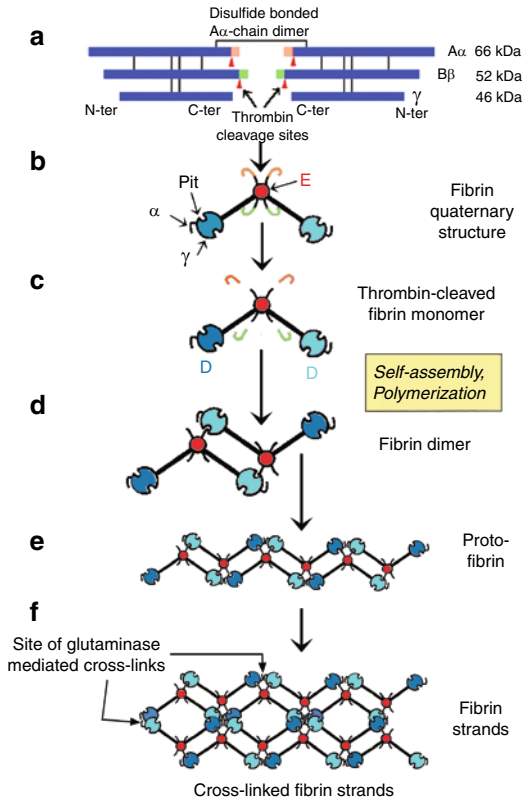


Fig. 11.9 Fibrinogen and fibrin structures. **(a) Fibrinogen polypeptides:** Three polypeptide chains are covalently linked by disulfide bonds. Each trimer is further head-to-head dimerized to form a hexamer whose molecular weight is ~330,000. **(b) Fibrinogen quaternary structure:** The N-terminal domains of the two Aα and two Bβ chains form an E node (letter E in red). The N-terminal 30 or so amino acids of each of these polypeptide folds out and around the outside of the E domain (pink and green). **(c) Fibrin activation:** Thrombin cleaves fibrinopeptides A (pink; 16 amino acid residues) and B (green; 14 amino acid residues) from fibrinogen. The loss of these fibrinopeptides exposes four knobs (black lines) that protrude above and below the E node. **(d) Fibrin dimerization:** The knobs protruding from the four cleaved polypeptides on the E node spontaneously adhere to pits in the D node of another molecule (blue). The D node is made from the C-terminal portion of the Bβ and most of the γ chain. The Aα chain forms a C-terminal flexible domain that lies away from the E domain and alongside the D to E linkage (Aα chain polar appendage). **(e) Polymerization:** The dimer initially lengthens by lateral addition of fibrin monomers to form thin fibrin strands (protofibrin polymers). **(f) Covalent cross-linking:** The transglutaminase reaction (see text) first cross-links close to the C-terminal ends of the γ chains on adjacent fibrin monomers (site indicated by γ in b above). A secondary site of cross-linking is between Aα chain polar appendages also indicated in b. This slower cross-linking reaction permits thick, antiparallel fibrin strands to develop. The chain also cross-links fibrin to additional proteins such as fibronectin exposed to the clot by the tissue damage (**a** – From Doug Tollefsen Lab Website, Fig. 10 slightly modified: (<http://tollefsen.wustl.edu/projects/coagulation/coagulation.html#III.D>), reproduced here with Dr. Tollefsen's permission). **b–f** – Reprinted from Fig. 4, slightly modified, from Fuss C, Palmaz JC, Sprague EA (2001) "Fibrinogen: structure, function, and surface interactions." J. Vasc. Intervent. Radiol. 12(8):677–682. Copyright 2001, with permission from Elsevier)

The genes for all three chains of fibrinogen are found within a 50-kb length of DNA on chromosome 4. Their DNA sequences show a high degree of homology, suggesting a common ancestral gene. The homology extends to sites upstream of the gene, suggesting a common set of regulatory elements to coordinate synthesis. The thrombin-cleaved fibrinopeptides pass around the bloodstream to the liver, where they induce the synthesis of more fibrinogen by inventory control feedback. Thus, fibrinogen that was consumed by clotting signals back to the liver that it needs replenishment in the blood.

11.4.2

Removal of a Blood Clot

A fibrin clot that has sealed an injured blood vessel must be removed (lysed) as the wound heals and new capillaries develop. Lysis is accomplished by plasmin, a serine protease derived from a soluble plasma protein precursor, plasminogen, yet another protein that is made in the liver and secreted into the blood. Plasminogen binds to lysine residues that protrude from the D nodes of fibrin but not fibrinogen. The binding causes plasminogen to attach an activator from the surrounding stromal tissues, *tissue plasminogen activator* (tPA) (Fig. 11.10a). tPA is a serine protease on the surface of endothelial cells and also secreted by them around sites of vascular injury. The attached tPA converts the bound plasminogen to plasmin by cutting its single polypeptide chain into two disulfide linked chains, similar to the cutting of prothrombin by factor Xa (Fig. 11.7). The newly formed plasmin dissociates and recognizes peptide bonds downstream of other lysine residues in the D–E node connecting region (Fig. 11.10a). The C-terminal lysine residues of partially hydrolyzed fibrin bind with greater affinity to plasminogen than the D node lysine residues, producing more plasmin and further accelerating fibrinolysis.

Plasmin is soluble but it remains active in the location of a clot. As it diffuses into the blood with clot fragments, the plasmin binds to α_2 -antiplasmin, a serine protease inhibitor (see next section). In addition to inhibiting plasmin in the blood (Fig. 11.10b), α_2 -antiplasmin inhibits various other serine proteases, especially activated protein C (APC) (next section) and elastase (Sect. 6.2.1). Plasmin action is inhibited where fibrin is cross-linked to fibronectin, but the large fibrin fragments tend to promote healing. The fragments of fibrin are named as shown in Fig. 11.10c and d. Factors that activate or inhibit fibrinolysis are summarized in Table 11.2.

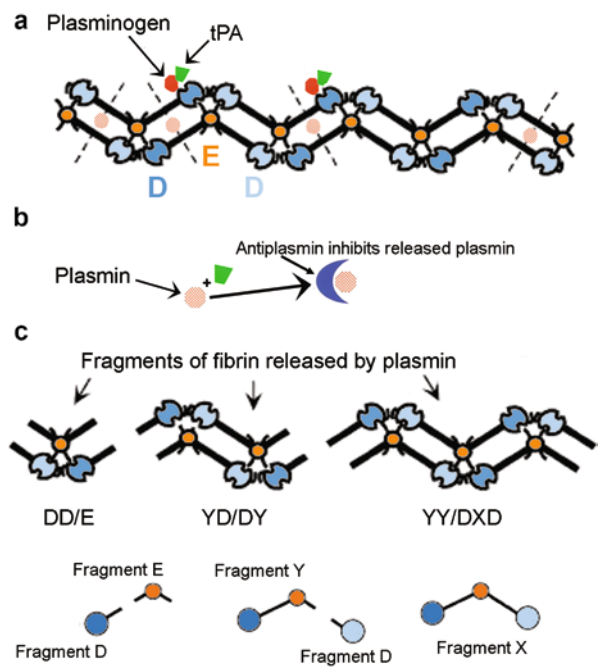


Fig. 11.10 Plasmin dissolution of fibrin. (a) *Plasminogen and tissue plasminogen activator (tPA) binding*: The D node in proto-fibrin and fibrin strands possesses lysine residues that protrude and bind plasma plasminogen (red hexagon). The attached plasminogen (but not free plasminogen) binds to tissue plasminogen activator released at the site of an injury (tPA – green) and is changed to plasmin (pink) by proteolysis. The activated plasmin is released but immediately binds to a nearby motif in the D to E linker region, which it hydrolyzes downstream of lysine residues (slanted broken lines). (b) *Plasmin inactivated*: The plasmin is now free to diffuse away and is subsequently inactivated by binding to antiplasmin (blue crescent) in the blood plasma. (c) *Fibrin fragments*: The fragments of fibrin can be the individual D or E nodes, a DE combination (fragment Y), or an E/DD combination (fragment X). Usually, the larger fragments shown are obtained because of incomplete fragmentation (see text) (Figure is derived by combining and slightly modifying Figs. 3 and 5, from Fuss C, Palmaz JC, Sprague EA (2001) “Fibrinogen: structure, function, and surface interactions.” J. Vasc. Intervent. Radiol. 12(8):677–682. Copyright 2001, with permission from Elsevier)

Table 11.2 Fibrinolysis and its inhibitors

Major fibrinolysis initiators	Major fibrinolysis inhibitors
Plasminogen/plasmin	α 2-Antiplasmin
Tissue plasminogen activator (tPA)/urokinase	Thrombin-activatable fibrinolysis inhibitor

Fibrinogen is a dimer of each of three interdigitated, parallel polypeptides folded into a dense central (N-terminal) and two outer (C-terminal) nodes. The central (E node) develops knobs after thrombin hydrolyzes off N-terminal peptides from two of the three polypeptide chains. The knobs spontaneously bind to pits in the D node of another fibrinogen or fibrin molecule and simultaneously activate a transglutaminase from blood plasma to covalently crosslink adjacent D nodes. The clot includes cross-linked stromal proteins from the injured region, especially fibronectin, and it shrinks, leaving a clear fluid called serum. The fibrinopeptides activate fibrinogen expression in the liver to replace the fibrinogen consumed by its conversion to fibrin. Fibrin is degraded by plasmin, a serine protease. Plasminogen is the plasmin precursor in blood plasma. It binds to lysine residues protruding from fibrin D nodes where it attaches tissue plasminogen activator (tPA), a serine proteinase secreted by vascular endothelium at sites of injury. The tPA cuts the bound plasminogen into two peptides that realign to form plasmin, which floats free and cuts peptide bonds downstream of other lysine residues between the D and E nodes. The C-terminal lysine residues activate more plasminogen, accelerating plasmin production and fibrinolysis. Plasmin that diffuses away from a clot is inhibited by plasma α_2 -antiplasmin, a nonspecific protease inhibitor.

11.5.1

Prevention of Unwanted Blood Clotting

Capillary damage causes platelets to secrete TXA_2 , an eicosanoid which activates them to promote coagulation (Sect. 11.2.1). If activated platelets escape the damaged region, they encounter another eicosanoid, prostaglandin I_2 (PGI_2) secreted by undamaged (healthy) endothelial cells. PGI_2 contains a unique double ring structure (Fig. 13.13) and is also called prostacyclin. PGI_2 binding to receptors on activated platelets stops TXA_2 secretion, collapses their fibrinogen-binding integrin, and removes their surface phosphatidylserine. Neither calcium ion-binding nor clotting cascade can develop (Fig. 11.11). PGI_2 also relaxes the smooth muscles of arterioles, keeping capillaries dilated and further reducing the risk of a clot forming in nearby undamaged regions (Sect. 13.5.5.).

If thrombin and factor Xa, the major activated blood coagulation factors (Fig. 11.6), escape into healthy blood vessels, blood clots will develop and occlude capillaries throughout the body. Direct inhibition of these activated enzymes in the blood flow utilizes *serine protease inhibitors*, of which there are two common types: a *Kunitz inhibitor* and a *serpin*. The former possess a *Kunitz domain*, a convex antiparallel β -sheet that exactly fits into the concave active site of a serine protease, directly blocking it (*lock and key mechanism*). By contrast, serpins undergo complex interactions with other proteins to cause conformational changes that *bait and block* the catalytic action (Fig. 11.12 shows the bait). Table 11.3 lists the major coagulation inhibitors and cofactors, their targets and mechanisms of action.

Thrombin and factor Xa that have escaped into the blood flow are both inhibited by serpins, *anti-thrombin III* (ATIII) and *heparin cofactor II* (HCII). These proteins bind to heparin sulfate or dermatan sulfate (Sect. 6.3.1), glycosaminoglycans which are secreted onto the luminal surface of healthy endothelial cells and also released into the blood from mast cells activated by an injury. Among the heparin molecules is a pentaglycan sequence

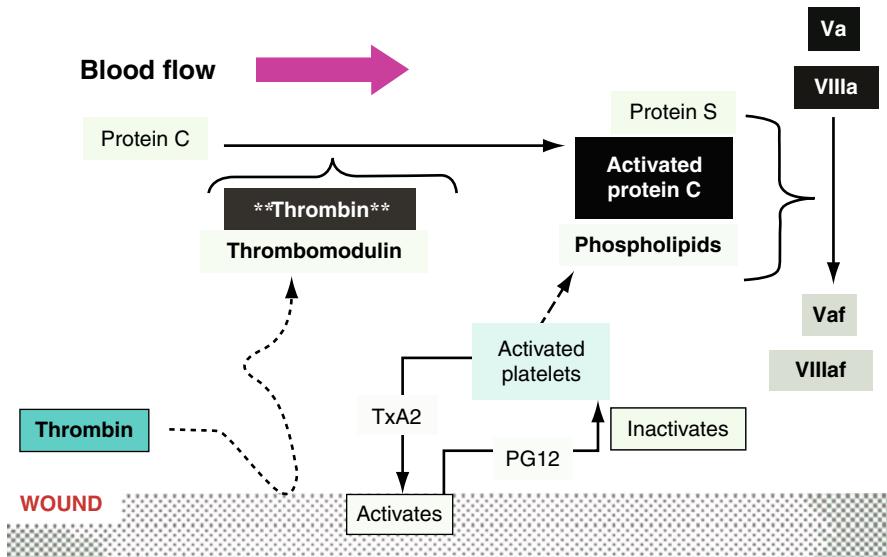


Fig. 11.11 Regulation of coagulation downstream from a wound *Bottom center*: Activated platelets secrete the prostaglandin small molecule thromboxane A₂ (TXA₂) to recruit and activate more platelets, except that healthy intact endothelium responds to TXA₂ by making prostaglandin I₂ (PGI₂) which inhibits recruitment away from the injury. *Top*: Thrombomodulin (TM) on the luminal surface of intact blood vessel endothelium binds thrombin and changes its specificity. Instead of activating fibrin, thrombin activates protein C, which adheres by a gla domain to activated platelets. Activated protein C activates plasma thrombin activatable fibrinolysis inhibitor (see text) and binds to protein S, another protease. The bound complex with protein S (*top right* of figure) inactivates factors Va and VIIIa by cutting each of them into two fragments (Vaf and VIIIaf) (Original figure submitted by Dr Paul DeAngelis, Department of Biochemistry, University of Oklahoma HSC, Oklahoma City, OK, USA)

noted in the legend to Fig. 11.12. This sequence attaches to ATIII in which it alters the conformation of a surface loop and causes an arginine residue to protrude (Fig. 11.12). Other residues on the same heparin polymer bind to exosite II on thrombin (Fig. 11.8) or a similar site on factor Xa (or IXa). The protruding arginine residue penetrates to the serine residue at the catalytic center of the attached thrombin (or factor Xa or factor IXa) where it inactivates the rate of proteolysis 300 to 1,000 fold (Fig. 11.12b). Once the ternary complex has formed, conformational changes keep ATIII bound to thrombin while releasing the heparin sulfate. Heparin cofactor II (HCII) binds to a different heparin motif or to dermatan sulfate. It undergoes similar changes, but a different amino acid protrudes from the homologous loop to inhibit thrombin or factor Xa.

The activity of surface-bound factor Xa is controlled by a third serpin, *Z-dependent protease inhibitor* (ZPI) which circulates in blood along with protein Z. The latter has a gla domain and it attaches to the activated platelet surface alongside the activated clotting factors. Protein Z attaches ZPI in which it alters the conformation of a surface loop and causes a residue, not arginine, to protrude and inactivate the bound factor Xa. Protein Z dissociates from the ZPI-factor Xa complex, but ZPI itself gets cut by factor Xa into two fragments that soon dissociate from the complex. Protein Z replaces a glycosaminoglycan in the previous mechanism, but ZPI inhibition of factor Xa is transient. ZPI may work best where an

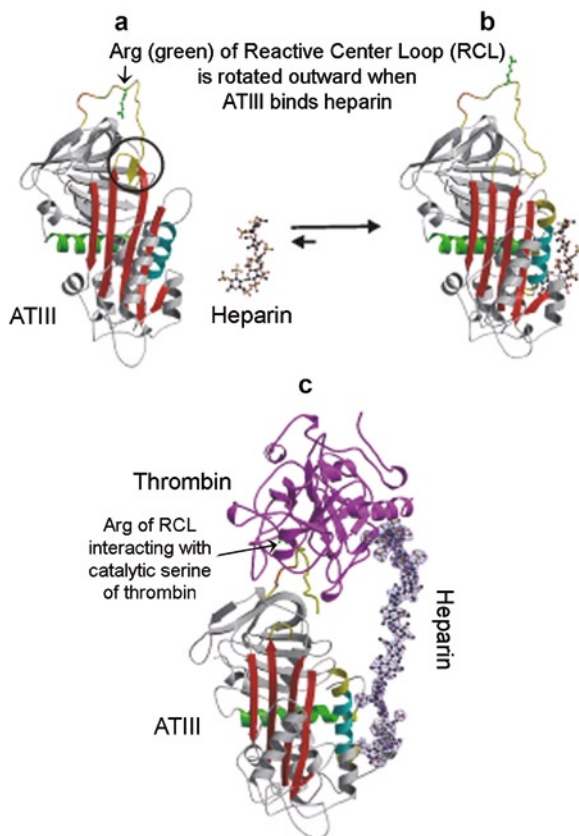


Fig. 11.12 Heparin catalysis of thrombin inhibition by antithrombin. **(a)** *Antithrombin and heparin pentasaccharide separately.* A hinge region (circled) expels a reactive center loop (RCL, yellow) from the bulk of the molecule (red, green and gray). The RCL has an arginine residue (green) that points inward, towards the bulk of the molecule. Heparin is to the right, a slightly curved glycan chain (blue and pink). **(b)** *Antithrombin and heparin pentasaccharide bound together.* ATIII binds strongly to heparin (large forward arrow). Binding causes the hinge region of ATIII to expel more RCL loop (yellow) from the central-sheet (red and gray) and the arginine residue (green) now points outward, away from central sheet region. **(c)** *The antithrombin-heparin-thrombin ternary complex.* Thrombin (magenta) binds by its glycan binding site (Fig. 11.8) to the heparin (blue and pink) attached to ATIII and the extended RCL (yellow) with its outward-pointing arginine residue (green) enters thrombin's catalytic serine residue (arrow) where it blocks thrombin's proteolytic action. Once the ternary complex has formed, conformational changes to both ATIII and thrombin release the heparin. (Adapted by permission from Li W, Johnson DJ, Esmon CT and Huntington JA (2004 Sept) "Structure of the antithrombin-thrombin-heparin ternary complex reveals the antithrombotic mechanism of heparin." *Nat. Struct. Mol. Biol.* 11(9):857–862. Copyright 2004, Macmillan Publishers Ltd.)

Table 11.3 Major coagulation inhibitors

Inhibitor	Target
Antithrombin III ^{a,b}	Thrombin ^a , Factor Xa ^a
Heparin cofactor II ^{a,b}	Thrombin ^a , Factor Xa ^a
Tissue factor pathway inhibitor (TFPI) ^c	TF-VIIa, Xa
Z-dependent protease inhibitor (ZPI) ^b	Xa on surface
Protein C ^d	Thrombin
Protein S ^e	Protein C
Protein Z ^e	ZPI

^aPossesses a glycosaminoglycan binding site

^bSerpin domain

^cKunitz domain

^dPossesses a gla domain and generates a protease

^ePossesses a gla domain without generating a protease

increased flow of blood provides more Z and ZPI proteins to bind and inhibit factor Xa attached to activated platelets or damaged cell membrane surfaces.

Tissue factor pathway inhibitor (TFPI) is a Kunitz type inhibitor composed of three Kunitz domains. The N-terminal Kunitz domain binds to and inhibits the VIIa-TF complex that activates factor X (Fig. 11.6a), whereas the downstream Kunitz domain binds directly to factor Xa and inhibits it strongly and permanently. No function has yet been demonstrated for the third Kunitz domain. The C-terminal region of TFPI is basic and remains attached to endothelial cell surfaces where it inhibits inadvertent factor Xa activation. Studies in mice indicate that knocking out the gene for TFPI stops fetal development. Indeed, clotting diseases such as a stroke and heart attacks are associated with mutations of ATIII, HCII and ZPI, but not of TFPI. Like TF (Sect. 11.3.1), diminished TFPI activity may be incompatible with life.

11.5.2

Thrombomodulin and Protein C and Protein S

If thrombin escapes the damaged region without being inactivated by the ATIII or HCII serpins, its glycan-binding site (exosite II) directly interacts with the glycosaminoglycans on the inner surface of an intact blood vessel. This interaction causes conformational changes to exosite I, which no longer binds fibrinogen, but instead binds to *thrombomodulin*, Tm (Fig 11.11). Tm is secreted by endothelial cells onto the capillary lumen surface alongside glycosaminoglycans and tPA (tPA is described in Sect. 11.4.2).

Once thrombin binds to Tm, the complex cuts two unrelated plasma proteins, thrombin activatable fibrinolysis inhibitor (TA fibrinolysis inhibitor) and protein C, instead of fibrinogen, transglutaminase and factors VIII and V (Sect. 11.3.4). The thrombin–Tm activated (cleaved) TA fibrinolysis inhibitor is a carboxypeptidase that removes the C-terminal lysine residues. These residues are present in fibrin D-E region fragments (Fig. 11.10) following initial plasmin action, and their loss prevents a rapid acceleration of fibrinolysis (Sect. 11.4.2).

Protein C (listed in Tables 11.1 and 11.3) is a vitamin K-dependent (VKD) protein with a multi-domain structure. It has a serine protease domain, two epidermal growth factor (EGF) domains, and a gla domain at its N terminus. The gla domain facilitates the Ca^{2+} ion-mediated coordination to phospholipids on activated endothelial cells and platelets, albeit with lower affinity than other vitamin K-dependent proteins. The thrombin–Tm complex activates protein C to become a serine protease (*activated protein C*, APC), which binds to another gla domain plasma protein, *protein S*. APC bound to protein S cleaves factors VIIIa and Va (making Vaf and VIIIaf), reducing the clotting signal (Fig. 11.11). The extent of this reduction is the reverse of its boosting by factors VIIIa and Va, $\sim 10^5$. Thus, the thrombin–Tm complex inhibits clot formation at the edges of the injured area by *changing the specificity of thrombin so that it cleaves and activates plasma protein C*.

Protein S was discovered because bleeding defects occur in some individuals whose blood clotting factors appear normal. Studies of their blood plasma proteins indicated that they were missing what became known as protein S. The absence of protein S prevents activated protein C from degrading factors VIIIa and Va. Because these helper factors remain up-regulated, individuals with absent or mutated protein S are unusually prone to strokes and heart attacks. In other individuals, excessive clotting is due to factor V and factor VIII mutations that prevent cleavage of the activated forms (Va and VIIIa) by the APC–protein S complex. The activation and inactivation of factors V and VIII differ because the respective activation sites use different proteases and different motifs. A mutation of the site at which APC–protein S cleaves factor Va (or VIIIa) results in excessive clotting, strokes and increased susceptibility to heart attacks, the same phenotype as a mutation in protein S.

Figure 11.13 summarizes the entire clotting process: initiation, major pathways, fibrin formation and fibrinolysis, clotting control pathways, and major associated diseases.

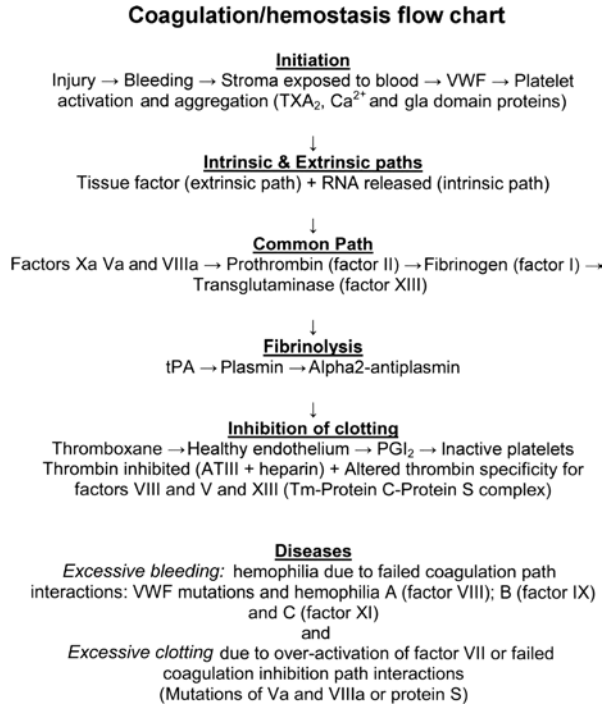
There are three processes that inactivate the clotting process: platelet inactivation by the eicosanoid system; thrombin inactivation by glycosaminoglycan/serpin (ATIII); and factors Va and VIIIa inactivation by the thrombomodulin/protein C/protein S system. Independently of their binding to protein C, thrombin–thrombomodulin complexes also inhibit fibrinolysis by binding to and activating a protein that removes the fibrin binding site for plasmin. The absence of protein S, or mutations that inhibit protein S binding to activated protein C, keep factors VIIIa and Va upregulated and cause excessive clotting and increased susceptibility to heart attacks. Mutations of factors VIIIa and Va that prevent their cleavage by the APC/protein S complex also cause excessive clotting. The clotting process and its controls are summarized in Fig. 11.13.

11.6.1

Drugs to Remove a Pathogenic Thrombus or Embolus: “Clot Busters”

Age- and diet-related degeneration of the artery intima (Sect. 11.1.1) results in fatty deposits that narrow an artery, predisposing to endothelial surface injury and clotting, *pathogenic thrombus formation*. A thrombus most often results in a myocardial infarction (heart attack) or stroke, but less dramatic effects occur from pathogenic thrombi in other organs. Alternatively, a clot may develop because of an irregular blood flow caused by a cardiac

Fig. 11.13 Coagulation flow chart. Blood coagulation events are divided into initiation, extrinsic and intrinsic path activation, and the common path to fibrin. Controls are fibrinolysis as healing begins and inhibition of clotting factors in a healthy (uninjured) blood vessel. Diseases are excessive bleeding (hemophilia) and excessive clotting (Original figure)



arrhythmia, or an excessive lack of movement, for example, of the legs during a long flight or at a computer. The irregular blood flow allows VWF aggregates to interact with and activate platelets away from the endothelium.

A clot that breaks free, *an embolus*, may become entrapped in a small artery or lodge in fine capillary beds, most often within the brain or lung, causing ischemia to the downstream region. Inhibition of the blood supply to the brain causes a stroke whose symptoms depend on the region affected. Inhibition of the oxygenation of hemoglobin in the lung causes a dry cough, shortness of breath or (rarely) sudden death. Moving around avoids a stagnant blood flow and frequent drinks of water keep the blood from being overconcentrated. Both these measures prevent an embolus from developing. Clots develop rapidly and must be broken up within an hour or 2 for serious tissue damage to be prevented. For example, the clot is usually in an artery and blocks oxygen and nutrients from the downstream region. If the artery is totally obstructed, all the downstream tissues will die from a lack of oxygen and nutrients. More commonly, there is a partial closure, and there is time to dissolve the clot before oxygen and nutrient deprivation kills the downstream cells.

Treatment requires a "clot buster" to remove the fibrin clot. Because plasminogen circulates in the blood in relatively large amounts, a common procedure is to give intravenous tissue plasminogen activator (tPA) to speed its conversion to plasmin (Fig. 11.10). Thus, intravenous tPA is the best therapy for acute symptoms of clot development. Because the half-life of infused tPA in blood plasma is only 4–5 min, it should be continuously infused for about an hour to dissolve enough clot to limit a potential ischemia. Recombinant human tPA is preferred to animal tPA because it is the natural protein and can be safely injected

into the blood stream (nonantigenic). Oral tPA cannot be absorbed because, like any other food, it is first digested to amino acids and small peptides.

Two other proteins, urokinase and streptokinase, are cheaper alternatives for tPA, but they activate plasminogen throughout the body, not just the fibrin-bound plasminogen like tPA (Fig. 11.10). Urokinase is a plasminogen activator that initiates plasmin degradation of the stroma following injury, infection or environmental stress (Sect. 8.1.3). Streptokinase is made by various pathogenic streptococcal bacteria and it enhances bacterial spread in the tissues by its streptokinase acting like urokinase to activate plasmin and degrade the stroma. Because urokinase and streptokinase do not restrict the plasminogen activation to fibrin, there is a greater risk of uncontrolled bleeding developing because of clotting failure. Subjects may also become resistant to streptokinase because it is a foreign protein. Sequential treatments will quickly induce antibodies that bind to and remove streptokinase from the blood before it can react with plasminogen.

11.6.2

Drugs That Inhibit Excessive Clot Formation

Widespread uncontrolled clotting (disseminated intravascular coagulation, DIC) is associated with infection, drug complications, allergies or various other diseases or conditions. In this instance, therapy is better if it prevents the widespread thrombin activation instead of dissolving the fibrin. As noted above, heparin is a potent anticoagulant that promotes thrombin interaction with antithrombin III (see Fig. 11.12 and Sect. 11.3.4). Low-molecular weight heparin is the safest to use because it binds to only one molecule of thrombin (factor IIa). The heparin is injected because, like tPA, it is not absorbed in intact form from the gut. Heparin is also used during surgery to prevent incisions from clotting until after stent placement or stitching is completed. An antidote to heparin is protamine sulfate, a positively charged small protein that complexes with heparin and prevents ATIII or thrombin binding.

11.6.3

Drugs That Retard Clot Formation

Vitamin K antagonists, most commonly the coumarins (coumadin and warfarin), are a set of anticoagulant drugs often referred to as “blood thinners.” These drugs structurally mimic vitamin K and prevent γ -carboxyglutamate (gla) formation (Sect. 11.2.2). The gla-containing coagulation factors localize by binding to Ca^{2+} ions on the surface of activated platelets (Fig. 11.4).

Coumarins act slowly because proteins that have already been synthesized with a gla domain (Table 11.1) must first be eliminated. The clotting factor half-life is 1–5 days, and so it takes about a week for the gla residue-containing proteins to decrease significantly. In the presence of a high concentration of vitamin K, high levels of dietary quinone (KH_2) are made and oxidized without an epoxide intermediate. Vitamin K therefore promotes gla formation in the presence of coumarins. Nevertheless, new protein has to be synthesized, post-translationally modified at the appropriate glutamate residues and secreted. Increasing the blood concentration of gla clotting factors is therefore slow also.

Coumarins are potentially highly toxic. Blood levels must be monitored carefully, and vitamin K must be given as necessary if overdosing is detected. An important concern is dehydration, which increases the drug concentration. Dehydration is especially common in elderly patients who are also often on a diuretic. In addition, a change in multivitamins may shift the correct dose of a coumarin. It is therefore especially important to communicate with both patient and supervising doctor before undertaking any dental surgery procedures on these patients.

11.6.4

Drugs That Inhibit Platelet Activation

Platelets use *cyclooxygenases* to make thromboxane A_2 for recruiting and activating other platelets (Sect. 11.2.1). Intact endothelial cells cyclooxygenase to make prostaglandin PGI_2 (Sect. 11.5.1). Because platelets are cell remnants and have no DNA or RNA, they cannot transcribe or translate new proteins. Thus, once the platelet cyclooxygenases are inhibited, the platelets cannot make more thromboxane to promote their activation. By contrast, PGI_2 -producing endothelial cells simply synthesize additional enzyme to make more PGI_2 (Fig. 11.11).

All cyclooxygenases (Classes 1 and 2) are irreversibly inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen. Thus, a daily low dose of aspirin inhibits clotting by reducing thromboxane levels from platelets, while interfering less with PGI_2 production. Because platelets are almost completely renewed weekly, aspirin dosage must be maintained. Ibuprofen does not inhibit platelet thromboxane production as effectively as aspirin (Sect. 13.5.5). Low doses of aspirin are preferable because high doses interfere with vitamin K uptake and promote excessive intestinal bleeding. Spontaneous bleeding can occur due to a lack of vitamin K, just as when an individual is given coumarin.

11.6.5

Drugs That Promote Clotting

After surgery, an excessive activation of plasmin can cause blood loss and inhibitors of plasmin are used to retard fibrinolysis. The drugs of choice are epsilon-aminocaproic acid and tranexamic acid, but the latter is preferred because smaller doses inhibit plasmin. Plasmin interacts with an amino acid motif between the D and E domains of fibrin (see Fig. 11.10). The motif contains a lysine residue that is N-terminal to the plasmin cleavage point and a substrate for plasmin binding. Thus, plasmin binds to fibrin by a lysine motif and cuts downstream (C-terminal) to its binding site. Tranexamic acid binds to plasmin and prevents it from binding to lysine residues on the motif; the clot is stabilized and no longer hydrolyzed.

Although slowing clot removal might seem dangerous, tranexamic acid does not affect the clotting process and is therefore relatively safe. If given intravenously for uncontrolled bleeding after surgery, a correct amount of antidotal heparin should be available and ready to inject intravenously in a syringe. Minutes may be important after an intravenous injection. Tranexamic acid is given orally when postsurgical bleeding is a problem or anticipated after surgery as in hemophiliacs. Tranexamic acid is rapidly absorbed and excreted, preventing the plasma concentration rising to dangerous levels.

Drugs to remove a pathogenic thrombus or embolus: Recombinant tissue plasminogen activator (tPA) breaks up fibrin clots by activating plasmin when intravenously infused for an hour. Urokinase and streptokinase are cheaper alternatives, but their global activation of plasminogen may cause uncontrolled bleeding. Subjects previously exposed to streptokinase may have antibodies that prevent its activation of plasminogen. *Drugs that inhibit excessive clotting:* Intravenous heparin possessing the pentaglycan activates ATIII to inactivate thrombin, factor Xa and IXa. *Drugs that retard clotting:* clot Coumarins and vitamin K antagonists slow thrombin production by inhibiting gla formation. Because inhibition and antidote (vitamin K) take a week to be effective, blood clotting efficacy must be monitored. *Drugs that inhibit platelet activation:* Cyclo-oxygenase inhibitors such as aspirin inhibit thromboxane A₄ production, slowing platelet activation. Prostaglandin I₂ production is also reduced, but the enzyme in endothelial cells is constantly being synthesized *de novo*, unlike the platelet enzyme which takes a week to replace. High doses of cyclooxygenase inhibitors interfere with vitamin K uptake and promote intestinal bleeding. *Drugs that promote clotting:* Major surgery, severe menstruation, and hemophilia overactivate plasmin, destabilizing fibrin clots. Inhibitors of plasmin are epsilon-aminocaproic acid or tranexamic acid which retard fibrin digestion by covering extruding lysine residues that otherwise promote plasminogen activation.

11.6.6

Laboratory Tests to Determine the State of the Blood Clotting System

Laboratory tests can identify a faulty component of clotting or whether an elderly patient is required to alter coumarin drug or vitamin K dosages before a tooth is extracted. Samples of the patient's blood are taken and treated in various ways to allow the observation of the appropriate steps in the hemostasis pathways. For example, tissue factor is added to the blood sample for the prothrombin time (PT) test. Alternatively, certain clotting system components in the blood sample are removed or inactivated. These tests can be run quickly on small samples of blood.

1. The prothrombin time (PT) test is measured by how long fresh blood clots after adding a fixed amount of tissue factor. This time (normally 11–14 s) is a measure of the rate of factor VII conversion to factor VIIa (extrinsic pathway). The test is usually done in children to determine a coagulation factor deficiency, or in adults monitor the dose of a coumarin blood thinner such as warfarin. Time is increased in individuals taking coumarins or exhibiting liver disease. Specialized tests are required to exclude liver disease. Time is shortened in disseminated intravascular coagulation, but again further tests are required (see item 4).
2. The activated partial thromboplastin time (APTT) measures the efficacy of the intrinsic and common pathways. Blood is collected and oxalate or citrate is added to arrest coagulation by binding calcium. In the laboratory, calcium is added to reverse the anticoagu-

lant effect of the oxalate and phospholipid. Silica, a synthetic contact phase, is then added to the plasma sample and mixed. The time for a thrombus (clot) to form takes 25–35 s. A shorter time has no meaning. A prolonged time occurs in hemophiliacs, but a bleeding time (BT) test (below) is also required to separate von Willebrand's disease from hemophilia A through C. If the patient is taking blood thinners, or an anticoagulant such as heparin, clotting takes up to two and a half times longer.

3. The bleeding time (BT) test is made from how long a small, precise skin wound requires to stop bleeding and measures platelet functions. A nick is made in the ear, and touched with a fresh piece of filter paper every 15 s until the bleeding stops. The count of how many 15 s intervals have passed indicates the bleeding time. Normal is six intervals of 15 s. More intervals indicate von Willebrand's disease, or thrombocytopenia (deficiency of platelets), or a patient taking aspirin or ibuprofen. Patients with hemophilia A through C or taking coumarin have a normal number of intervals because these conditions do not affect platelet plug formation.

A new method is replacing the BT assay. A *platelet function analyzer* simulates the exposure of blood to a vascular wall injury. Instead of a nick in the ear, blood is collected by venipuncture into a tube containing 3.8% citrate (anticoagulant) and aspirated from a reservoir through a capillary to a membrane coated with collagen and epinephrine or ADP (Sect.11.2.1). The membrane contains a central aperture that simulates a blood vessel wall injury. As blood flows through the aperture, the platelets adhere and aggregate. The time until the aperture is completely occluded by the platelet/red blood cell thrombus, is the *closure time*. The membrane coat and amount of citrate affects the closure time. For a collagen/epinephrine aperture, the bleeding time is normally about 2 min for 3.2% citrated blood and 2.5 min for 3.8%.

(1) Prothrombin time test determines intrinsic factor VIIa activation, which is gla-activated so that the time of the test increased by coumarin; (2) The activated partial thromboplastin time measures the efficacy of the intrinsic and common pathways, which are prolonged in hemophiliacs, von Willebrand's disease or when heparin or coumarin therapy is given; (3) The bleeding time is longer in von Willebrand's disease and thrombocytopenia, or during low dose NSAIDS therapy, but normal in the presence of coumarin and in hemophilia A through C; (4) An increased thrombin clotting time indicates a lack of fibrinogen or slower rate of fibrin clot development, usually due to liver disease (detectable in other ways), but occasionally to genetic mutations. A faster thrombin clotting time may indicate disseminated vascular thrombosis and is confirmed by the presence of abnormally high fibrinopeptide levels.

This chapter briefly describes the key elements of salivary gland structure, their products, and their various functions, concluding with a discussion of how they control the oral microbiota in conjunction with oral hygiene (Sect. 1). Following a brief discussion of secretion (Sect. 2), there is a detailed description of the mucous proteins and their glycans (Sect. 3) and their relationship to glycans at the red blood cell surface (Sect. 4). The chapter concludes with a detailed description of the structure and functioning of the salivary amylase (Sect. 5) and its proline-rich proteins and salivary agglutinin (Sect. 6).

12.1.1. Cell Biology of Salivary Glands

Saliva is secreted into the oral cavity by pairs of major glands, the parotid, submandibular, and sublingual (Fig. 12.1a), and by many minor glands scattered throughout the oral epithelium. Each gland is composed of clusters of epithelial-like cells, *acinar cells* secreting a serous (watery) *fluid* or *tubular cells* secreting a *mucous* (viscous) fluid (Fig. 12.1b). Parotid glands are composed only of acinar cells; sublingual and submandibular glands have both types (mixed). Each cluster ends with a small duct that, in the major glands, joins up to a common collection duct and empties into the oral cavity from the upper, posterior region of each cheek (parotid), or on either side of the frenum beneath the tongue (submandibular and sublingual). Minor glands are composed of a single acinus or tubule cluster, mostly serous at the lips and increasingly mucous towards the back of the mouth.

12.1.2. Whole Saliva: Collection and Composition

Whole saliva is collected by asking volunteers to spit into an ice-cooled vial. If necessary, the flow is stimulated by chewing paraffin wax or washed elastic bands. Pure parotid gland secretions are collected using a Lashley Cup, a small plastic cup held against the duct orifice inside the cheek by a vacuum. The secretion drains into a vial through a tube that

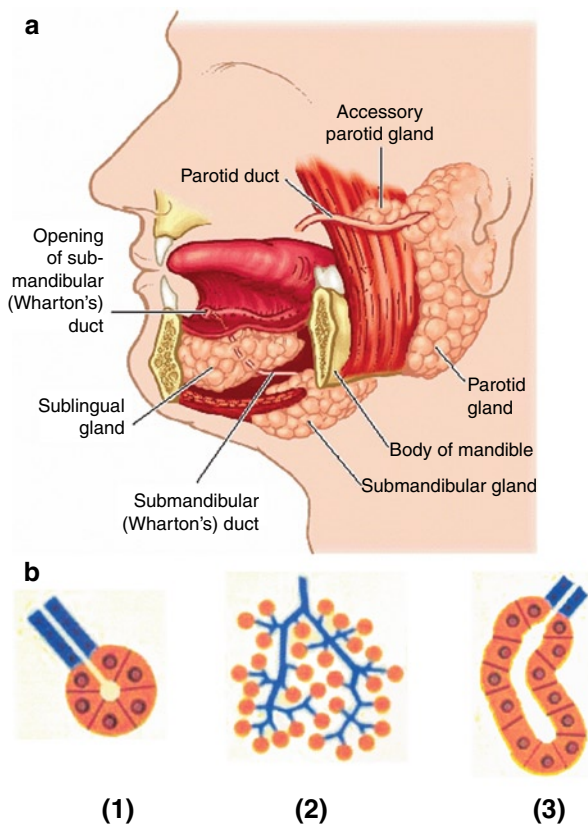


Fig. 12.1 The major salivary glands: **(a) Anatomy:** The parotid gland lies under the ear and behind the back of the mandible. The duct excretes its contents bilaterally into the buccal mucosa opposite the second upper molars. The sublingual gland lies beneath the tongue and the submandibular gland curls around the lower inside region of the mandible, mostly forward of the angle. The ducts from these two glands release their secretions close together bilaterally beneath the tongue. **(b) Composition:** The salivary glands are exocrine glands that are composed of serous cells around a single acinus as in a minor gland (1), or group of acini sharing a common collection duct as in a major gland (2), or mucus cells around a single tubule (3), or group of tubules (similar to acini except that cells around the collection duct are in a tubular arrangement; not shown). **(a):** Figure from Dorland's Illustrated Medical Dictionary. 31st ed. Elsevier Saunders: 2007, p 790. Figure, "Salivary Glands," under "Glands" Online at http://medicaldictionary.thefreedictionary.com/_/viewer.aspx?path=dorland&name=gland_salivary.jpg; **b:** Figure from <http://www.siumed.edu/~dking2/erg/glands.htm#acini>

fits into a hole in the cup. The flow is stimulated by sucking a sour lemon drop or by applying a drop of sodium citrate to the tongue. The collection of pure submandibular, sublingual, or minor gland secretions requires individually designed equipment.

Whole saliva is a dilute, viscous solution containing electrolytes, proteins, and epithelial cells from the oral mucosa. The major electrolytes are sodium chloride and

sodium bicarbonate. Mucin proteins mediate the viscosity, saliva's most obvious characteristic. Mucins are proteoglycans with many short, negatively charged side-chains, and they are made only by mucous cells: 5-15% of proteins from the submandibular gland, 10-30% of proteins from the sublingual gland, and virtually all the proteins from minor mucous glands. Besides mucins, the major proteins in saliva are α -amylase (α 1,4 glucan endohydrolase) and *proline-rich proteins*. The salivary glands secrete α -amylase into blood as well as saliva. Salivary α -amylase is encoded by a single gene (*Amyl*) on chromosome 1, but traces of enzymes in saliva modify it, so that a mixture of proteins of similar size and charge (α -amylase isozyme families) appears on gel electrophoresis. The proline-rich proteins are encoded as one of two or three different alleles on each of six adjacent genes on chromosome 12 and also undergo proteolysis in saliva. They therefore give rise to an extremely complex mixture of small polypeptides on gel electrophoresis. Mucins are encoded by *MUC5B* on chromosome 11 and *MUC7* on chromosome 4.

In an individual maintaining oral hygiene, the total protein content of saliva is ~1.6 mg/mL, much less than in blood plasma. About half the proteins are amylase, 40-45% are proline-rich proteins, and 5-10% are mucins. Stimulated saliva has 20% less protein, but up to a tenfold greater sodium chloride content and up to double the sodium bicarbonate content. Bicarbonate is produced by the same intracellular carbonic anhydrase in acinar cells as in osteoclasts and red blood cells (Chap. 10). The greater sodium bicarbonate content of stimulated saliva makes its pH more alkaline (7.4–7.8) than unstimulated saliva (6.8–7.2).

The salivary glands also secrete urea, which some oral bacteria convert to ammonia and carbon dioxide with an enzyme, *urease*. The greater content of ammonia results in the oral cavity being better buffered to acids and better protection from caries (Chap. 15, Sect. 3). Calcium and phosphate are also present in saliva at supersaturating concentrations but do not precipitate due to protein chelation. Whole saliva also contains small amounts of various other proteins: *proteases*, *protease inhibitors* (*cystatins*), *type IV carbonic anhydrase*, *statherin*, *histatins*, *lysozyme*, *salivary agglutinin*, and *immunoglobulin A*.

Finally, the salivary glands concentrate sodium nitrate from fruit and vegetables. The sodium nitrate is absorbed into the blood plasma and concentrated 10 – 20 fold by salivary gland cells before secretion. The nitrate in saliva is reduced to sodium nitrite by *Eikenella corrodens*, a common commensal bacterium in human saliva (Sect. 12.1.5). Sodium nitrite is an important vasodilator, but it cannot cross healthy tissues.

12.1.3. Functions of the Salivary Components

If a major salivary gland is lost from trauma or disease, or if nasal allergies or sinus infections cause persistent mouth-breathing, or if tobacco smoking persists, the oral cavity becomes dry (xerostomia). The oral mucosa and teeth become covered with bacteria and dental caries and periodontal disease become difficult to control. The functions of whole

saliva are (1) lubricating the oral tissues and food particles; (2) promoting the clearance of food particles; (3) protecting the oral mucosa and teeth from excessive bacterial colonization; (4) forming an acquired pellicle that protects teeth surfaces from dissolution or over-accretion; and (5) stabilizing teeth surfaces from bacterial acid dissolution. The first three functions are performed mainly by the mucin and water content, aided by amylase and immunity proteins described later, the fourth is associated with other secreted salivary proteins, statherin, and the proline rich proteins, and the last is caused by buffers in saliva, mostly sodium bicarbonate (Table 12.1).

The stability of the tooth surface to spontaneous dissolution and accretion is primarily mediated by *statherin*, a 43 amino acid polypeptide encoded by a single gene (*Stath*) on chromosome 4. Its two N-terminal serine residues spontaneously become phosphorylated in saliva, and they attach statherin tightly to exposed tooth enamel, preventing dissolution or calcium phosphate accretion. Nevertheless, all proteins in *whole saliva* adhere to some extent to enamel, forming an *acquired pellicle* that replaces abraded *enamel cuticle* (Chap. 9). Differences in amount or composition of the proteins in whole saliva cause differences in acquired pellicle composition.

Carbonic anhydrase and *sodium bicarbonate* together neutralize the acids produced by bacterial metabolism of dietary carbohydrate (Fig. 12.2). When salivary carbonic anhydrase is swallowed, it adheres to the mucosal surface of the stomach where it remains active and forms carbonic acid from sodium bicarbonate in the gastric mucosa. A lack of salivary carbonic anhydrase causes acid to remain longer in the stomach, contributing to peptic disease in addition to dental caries (Sect. 15.3.3).

Table 12.1 Functions of saliva effector molecules

Functions	Effector molecules
Lubricate oral mucosa and food particles and inhibit bacterial colonization	Mucins
Facilitates clearance and inhibits bacterial colonization	Water (dilute secretion)
Digests starch in food particles, promoting bolus cohesion	Amylase
Stabilize tooth surface	Sodium bicarbonate, carbonic anhydrase, and statherin
Form acquired enamel pellicle	Proteins
Innate immunity that inhibits bacterial growth	Peroxidases, histatins, lysozyme, and lactoferrin
Innate immunity that enhances bacterial clearance	Salivary agglutinin and mucin
Acquired immunity that enhances bacterial clearance	Secretory immunoglobulin (sIgA)

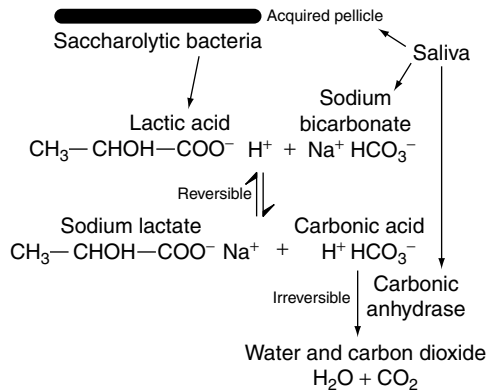


Fig. 12.2 Neutralization of bacterial acids by salivary carbonic anhydrase: Saliva contains proteins that form the acquired pellicle to which saccharolytic bacteria attach and grow anaerobically using dietary carbohydrate as substrate. Lactic acid is produced and reacts reversibly with salivary sodium bicarbonate to make sodium lactate and carbonic acid. Traces of salivary carbonic anhydrase in saliva contribute to the acquired pellicle and also remain in solution. The enzyme converts carbonic acid to water and carbon dioxide. This step is irreversible because carbon dioxide is lost to the environment whenever the mouth is opened. Thus, lactic acid is transformed into its salt, sodium lactate. (Original Figure)

12.1.4. Innate and Acquired Immune Proteins in Saliva

Innate immunity in saliva is mediated by enzymes, *peroxidase*, *lactoferrin* and *lysozyme*, by small peptides *histatins* and *proline-rich proteins*, and by two proteins, *salivary agglutinin* and *mucin MG2*. All are secreted, but the enzymes may be added independently from leukocytes at the gingival sulcus (Sects. 13.2.3. and 13.2.4). The peroxidase reaction is discussed Chap. 16, Fig. 16.8b. In saliva the cofactor (glutathione) is absent, and the electron-deficient oxygen species released by enzyme action convert chloride (Cl^-) and thiocyanate (SCN^-) ions to hypochlorite (OCl^-) and hypothiocyanate (OSCN^-) ions which diffuse into bacteria and inhibit their growth or kill them directly. Lactoferrin is encoded by chromosome 3 and secreted as an aggregate of four identical polypeptides surrounding two ferric (Fe^{3+}) ions. It is electron deficient like hypochlorite or hypothiocyanate and retards the colonization of Gram-negative bacteria and fungi on the oral mucosa. Lysozyme is a glycoside hydrolase encoded by *Lyz* on chromosome 12. It lyses bacteria by hydrolyzing a glycan bond of peptidoglycan on the surface of Gram-positive bacteria (Sect. 1.4.1) and is distantly related to amylase (GH family 22, Sect. 12.5.1.). Gram-negative bacteria are not readily hydrolyzed by this enzyme because a second, outer membrane covers the peptidoglycan (Sect. 1.4.1).

Histatins are positively charged small polypeptides encoded by two genes on chromosome 2, *His1* and *His2*. They bind to the oral mucosa and teeth where they inhibit bacterial

growth. The proline-rich proteins (PRPs) may be positively or negatively charged. The latter, the acidic PRPs, bind to teeth where they likely alter dental biofilm development (Sect. 12.6.1). Salivary agglutinin binds to various oral bacteria and causes them to be swallowed instead of forming biofilms. Mucin MG2 (Sect. 12.3.1) clears fungi similarly.

Acquired immunity is mediated by lymphocytes directly or by their secreting *immunoglobulins* (Ig). An acquired immune response in the oral cavity is induced by antigens, usually a foreign molecule from a bacterium or fungus that colonizes the oral cavity from the environment. High molecular weight surface components of these organisms activate *gut-associated lymphoid tracts* (GALT), which include the tonsils of the pharynx. Lymphoid cells within these tissues are induced to secrete IgA into the surrounding blood plasma, which passes into saliva and other secretions. In saliva, secreted IgA (sIgA) binds to the antigen that induced its production. The IgA–antigen complex is swallowed and digested in the stomach or small intestine.

12.1.5.

Poor Oral Hygiene Adds Bacteria and Host Leukocyte Products to Saliva

The bacteria in a healthy oral cavity, the *commensal oral microbiota*, protect it from disease by repelling disease-causing (pathogenic) bacteria. A weak point in this protection is adherence of the commensal microbiota to the acquired pellicle. *Microbial biofilms* attach to the teeth, and differences in salivary protein composition cause differences in which bacteria make up the commensal oral microbiota. This microbiota extends into the gingival sulcus and induces *gingival crevicular fluid* (GCF), an inflammatory exudate derived from blood plasma containing activated leukocytes (Sect. 13.1.2). Thus, differences in salivary composition influence how much GCF is produced. The GCF is a better substrate for microbial growth than saliva, and the presence of commensal biofilms on teeth surfaces provides a more favorable environment for the colonization by pathogenic bacteria that are otherwise present in only trace amounts. These bacteria are gram negative and asaccharolytic. They release proteases and cell surface components into the oral cavity along with asaccharolytic metabolic end products, ammonia, amines, and sulfides (Sect. 1.3.1), which give mixed whole saliva (spit) an offensive odor.

Whole saliva (spit) is a dilute, viscous solution of proteins and shed epithelial cells. The major electrolytes are sodium, chloride, and bicarbonate. Calcium and phosphate are present at a supersaturated concentration. Viscosity is due to mucins, proteoglycans with numerous short glycan chains that lubricate the oral cavity, hold a bolus of chewed food together, and reduce bacterial adherence to teeth. Besides mucins, the major proteins secreted in saliva are amylase and proline-rich proteins. The major electrolytes, sodium chloride and sodium bicarbonate, increase with stimulation of salivary flow, but the protein content decreases. All proteins in whole saliva adhere to some extent to

enamel, forming an acquired pellicle that replaces abraded enamel cuticle. Differences in the amount or composition of whole saliva may result in individual, saliva-associated differences in dental caries. Small amounts of carbonic anhydrase protect the teeth from bacterial acid dissolution. Small amounts of statherin are secreted to protect the teeth from accreting salivary calcium phosphate. Various innate and acquired immunity proteins protect from bacterial infections. Innate immunity in secreted saliva is mediated by enzymes, peroxidase, lactoferrin and lysozyme, by small peptides, histatins and proline-rich proteins, and by two proteins, salivary agglutinin and mucin MG2. Acquired immunity is mediated by IgA. A bacterial biofilm (plaque) develops on the acquired pellicle where it induces the exudation of gingival crevicular fluid from underlying capillaries. Asaccharolytic bacteria grow on this fluid and add malodorous metabolic end products to whole saliva.

12.2.1. Physiology and Biochemistry of Saliva Secretion

In the cytosol, polypeptides destined for secretion in saliva are steered to the cytosolic side of the rough *endoplasmic reticulum* (ER) by the amino acid sequence of their N-terminal domains. This domain interacts with a *signal recognition particle* (SRP) in the cytosol and stops translation until the ribosomal/SRP complex moves to a ribosomal receptor. Placement on the receptor releases the SRP particle and allows the ribosome to resume translation. Polypeptide synthesis is completed in the ER lumen instead of the cytosol, and moved to the smooth ER, where certain asparagine amide residues are glycosylated (N-linked glycosylation). The glycan is preformed attached to dolichol diphosphate in the cytosol and transferred to the asparagine amide acceptor after switching from cytosolic to luminal face of the ER (Fig. 12.3, upper third). Protein-rich vesicles form and are transported to the cis face of the Golgi (Fig. 12.3, lower two-thirds). As the proteins pass through the Golgi, glycosidases and glycan synthetases greatly alter the N-linked glycans and attach other glycans to the –OH group of certain serine and threonine residues in the protein (O-linked glycosylation, Fig. 12.3, lower two-thirds).

Vesicles containing proteins destined for intracellular use in lysosomes or outer membranes bud off from the *trans* face of the Golgi. Depending on their contents, some of the vesicles are diverted to nonsecretory vesicles by the presence of phosphomannose residues on their N-linked glycans or by possessing domains rich in hydrophobic amino acids. At the apical surface of salivary acini, the remaining vesicles accumulate as secretory vesicles. These vesicles become surrounded by myofibrils, which move the secretory vesicles to the cell membrane where they fuse and expel the saliva secretion into a small duct. The odor or taste of food provides a neuronal stimulus to the gland's myofibrils, and this stimulates salivary secretion.

Water is secreted separately. Neurotransmitters activate a 28-kDa integral membrane protein, aquaporin-5 (AQP5), one of 13 aquaporins in mammals. AQP5 is localized in lipid rafts in the plasma membrane of salivary cells and translocates to the apical plasma

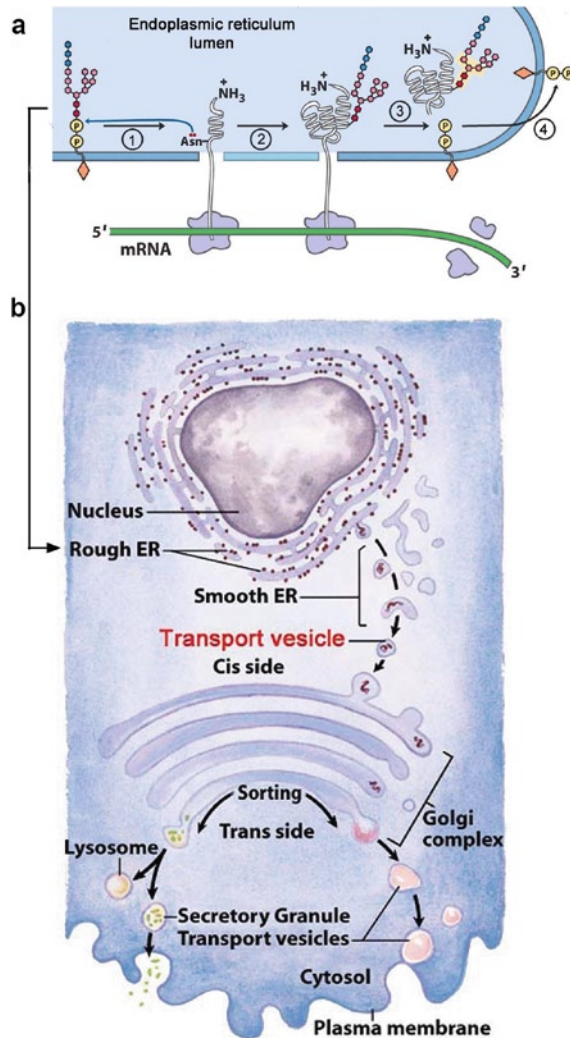


Fig. 12.3 How salivary proteins are secreted (a): N-linked glycans are synthesized and attached to dolichol diphosphate (orange diamond) in the cytosol. The completed glycan is translocated to the lumen of the endoplasmic reticulum (blue). The glycan attached to dolichol diphosphate by its C₁ (anomeric or reducing OH group) is *N*-acetyl glucosamine (GlcNAc; small red hexagon). The completed glycan also contains mannose and glucose residues (small pink and blue hexagons). (1) The glycan (oligopolysaccharide) is transferred to an asparagine residue of the growing peptide also in the lumen of the rough endoplasmic reticulum (ER). (2) Synthesis of the glycan-attached polypeptide amino acid sequence is completed. (3) The free dolichol diphosphate translocates back into the cytosol where a phosphatase removes its outer phosphate residue. (4) In the cytosol, a molecule of UDP-GlcNAc reattaches to give dolichol diphosphate GlcNAc (not shown) onto which an identical set of monosaccharides are added to give another glycan to be transferred as shown in 1. (b): The released glycan-attached polypeptide buds off into vesicles (smooth endoplasmic reticulum, ER) that discharge their contents into the cis-Golgi where the glycans are processed by removing most of the mannose and adding fucose, sialic acid, galactose, and other glycan residues. Mucin polypeptides have a sequence that activates an addition of glycans beginning with *N*-acetyl galactosamine (GalNAc) to serine and threonine –OH groups in the Golgi. In the trans-Golgi, the contents are either moved to lysosomal vesicles or secreted. (Modified from Figs. 27.34 and 27.35 in Lehninger Principles of Biochemistry. D.L. Nelson & M.M. Cox, 4th Ed. 2005. W.H. Freeman & Co., NY)

membrane where the secretory vesicles have accumulated. The extent of secretory vesicle and AQP5 translocation and fusion with the apical membrane depends on the extent of stimulation by cholinergic (acetylcholine) receptors, or α -1 (adrenergic) receptors coupled to G proteins and phospholipase C. Cholinergic receptors activate Ca^{2+} mobilizing receptors and signal both secretory vesicles (protein) and water secretion from salivary glands. Adrenergic stimulation of cyclic adenosine monophosphate (cAMP-dependent protein kinase system) increases secretory vesicle exocytosis immediately and the Ca^{2+} mobilizing receptors for water secretion afterward. The reason why these two neuronal activation responses are linked is not known. Eating and smelling food, or even just thinking of these things, transports water faster than the secretory vesicle contents; stimulated saliva has more water and less protein.

Salivary proteins are secreted like other proteins. Polypeptides destined for secretion are relocated by their N-terminal sequence to a ribosomal receptor on the cytosolic side of the endoplasmic reticular (ER) membrane. The polypeptides enter the ER lumen and move from rough to smooth ER where asparagine amide residues are glycosylated with a preformed glycan (N-linked glycosylation). Vesicles bud off and are transported to the Golgi where glycosidases and glycan synthetases modify the N-linked glycans and attach glycans to the -OH groups of serine and threonine residues (O-linked glycosylation). Water is separately secreted through a 28-kDa integral membrane protein, aquaporin (AQP5). AQP5 translocates from lipid rafts in the plasma membrane to the apical membrane where the secretory vesicles have accumulated and become surrounded by myofibrils. Smelling or ingesting food stimulates saliva, making it more dilute. The smell or ingestion activates cholinergic and adrenergic receptors at autonomic nerve endings around the salivary acini, causing faster AQP5 translocation through the cell membrane. Water is thus transported faster and independently of secretory vesicle contents, which are more forcibly expelled by the stronger myofibril contractions.

12.3.1. Salivary Mucin Composition

Mucin proteins account for the high viscosity of saliva in the oral cavity and, like the connective tissue proteoglycans, have many serine and threonine-linked glycan residues that absorb a large volume of water. A sulfate ($-\text{SO}_4$)⁻¹ group is also attached in the Golgi to the -OH group of certain glycan residues following synthesis of the chain, usually galactose, *N*-acetylgalactosamine or *N*-acetylglucosamine. Sulfation also accounts for the negative charge of the connective tissue proteoglycans (dermatan sulfate, chondroitin sulfate, etc.). Sialic acid residues terminate some of the short mucin glycan chains and contribute also to the strong net negative charge of salivary and other mucins. The connective tissue proteoglycan chains are longer and fewer and use uronate instead of sialate as the negatively charged glycan.

Human submandibular and sublingual glands secrete two mucin glycoproteins, MG1 and MG2. MG1 is a very large polypeptide composed of more than 5,500 amino acid residues (mol wt ~590 kDa). Its N- and C-terminal regions each contain two N-glycosylated sites (Fig. 12.4a) and a long central region of 72 repeating domains that are rich in threonine and

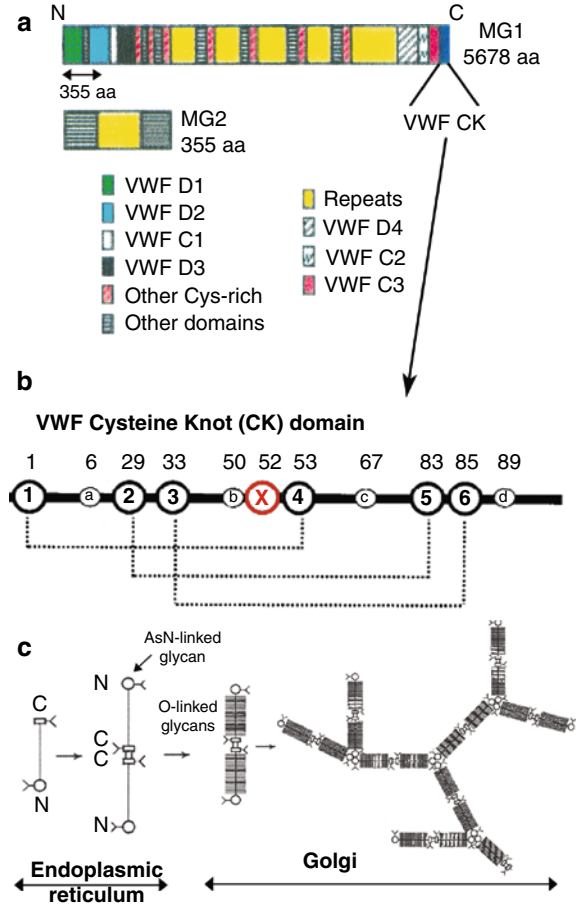


Fig. 12.4 Human salivary mucin composition. **(a)** *Polypeptide domains of MG1 and MG2.* Numbering begins after the signal sequence is removed in the endoplasmic reticulum before processing (see Expasy sequence Q9HC84, MUC5B_HUMAN). Both mucins have a central domain consisting of multiple short tandem repeats of a sequence that is rich in serine, threonine, and proline (yellow). Many of the serine and threonine residues become O-glycosylated (illustrated in C) and the proline residues keep the chain extended. The nonrepeat domains of MG1 at the N- and C-termini are mostly derived from domains of Von Willebrand factor (VWF), a protein associated with the blood clotting system. MG2 is much smaller, as illustrated by the *double-headed arrow* below the N-terminal region of MG1 (see text) and has no VWF domains. **(b)** *Cysteine knot (CK) domain of MG1.* In MG1, this domain consists of 89 amino acids that terminate shortly before the C-terminus. It includes six cysteines arranged in a knot-like topology. All of the cysteine residues in the domain are circled and numbered. Disulfide bonds between the second and fifth cysteines, and between the third and sixth cysteines, form a double ring that is penetrated by a disulfide bond between the first and fourth cysteines (the knot, *dotted lines*). Other cysteine residues (labeled a, b, c, and d) are present in MG1, VWF, and mucins from the pulmonary and digestive tracts, but absent from cystine knot-proteins. Within the knot, there is always a seventh unpaired cysteine residue (C^x red) that causes dimerization. In MG1, the knot domain (dark blue in A) is at the C-terminus. Two additional cysteine residues (not shown) are present in the cysteine knot of platelet derived growth

serine residues. Within this central region, the sequence TXXP (X = any small side-chain amino acid), recurs as a common threonine attachment site. The polypeptide is extended, due to the proline residues in the sequence, but flexible due to absence of collagen helix. The terminal -OH groups of the threonine and serine residues stick out and, in the Golgi, bind to a small family of enzymes that attach monosaccharides in an orderly manner.

The N- and C-terminal regions are less extended. They each possess a single N-linked glycan and are free of O-linked glycans. The C-terminus possesses a cysteine knot domain identical to that in von Willebrand factor and TGF- β (Fig. 12.4b) and therefore a free cysteine residue (Chapter 3, section 3.2.2.) which causes dimerization at the C-terminus of MG1 molecules (Fig. 12.4c). The D1, D2 and D3 domains near the N-terminus each possess a free cysteine residue, enabling each MG1 dimer to form a cystine bond with two other dimers until the molecule attains a molecular mass of about 25 million daltons (Fig. 12.4c). Although cysteine knot-aggregated VWF plays an important role in initiating blood clotting (Sect. 11.2.1), salivary mucins do not seem to enhance blood clotting in humans.

Mucin MG2 is less than a tenth the size of MG1, 355 amino acids; mol wt ~39 kDa. It consists of a single repeat domain with separate N- and C-terminal domains that are not VWF-related. The N-terminal domain has five cysteine residues, but the C-terminal domain has none and the secreted protein is probably a mixture of monomers and head-to-head disulfide dimers.

The gene for MG1 is called *MUC5B* (*MUC* for mucin) and the gene for MG2 is called *MUC7*. The gene for MG1 is called *MUC5B* (*MUC* for mucin) and the gene for MG2 is called *MUC7*. MG1 is made by mucous cells, whereas MG2 is made only by serous cells within mixed glands, i.e. only the submandibular and sublingual glands. The parotid and minor glands that contain only serous cells do not secrete either MG1 or MG2, whereas goblet cells of the intestinal and bronchial tracts secrete both MG1 and MG2. Other *MUC* genes encode similar mucins that protect the stomach from acid and the lungs from inhaled particles.

12.3.2.

Glycan Composition of Salivary Mucins

MG1 and MG2 account for the smooth surface of the oral mucosa and the viscosity of whole saliva. There are four monosaccharides associated with these mucins, of which three are illustrated in Fig. 12.5: *N*-acetyl galactosamine, L-fucose (6-deoxy-L-galactose),



Fig. 12.4 (continued) factor, another protein associated with blood clotting like VWF as discussed in Chap. 11. (c) *Assembly of human MG1*. MG1 is glycosylated and forms C-terminus to C-terminus cystine cross-links in the smooth ER (*left two diagrams*). It is then transported to the Golgi where its many repeat domains are glycosylated. In addition, the dimers develop crosslinked multimers using the free cysteine residues at its various N-terminal VWF D domains (*far right diagram*). (a) and (c) Modified Figs. 1 and 2 in Perez-Vilar J, Hill RL (1999 Nov 5) "The Structure and Assembly of Secreted Mucins." *J. Biol. Chem.* 271(45), 31751–4. (b) Slightly modified from Fig. 1 in Bell SL, Xu, G and Forstner, JF. (2001) "Role of the cystine-knot motif at the C-terminus of rat mucin protein." *Biochem. J.* 357: 203–209. Reproduced with permission from Portland Press).

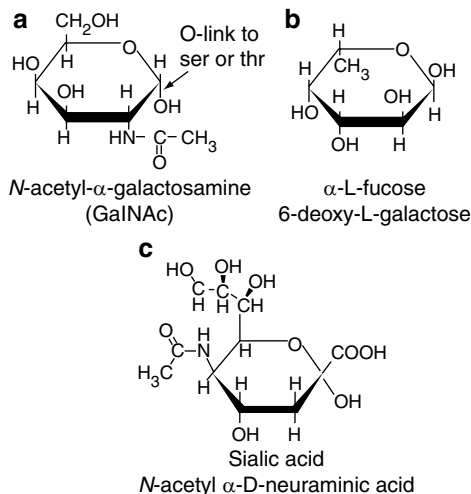
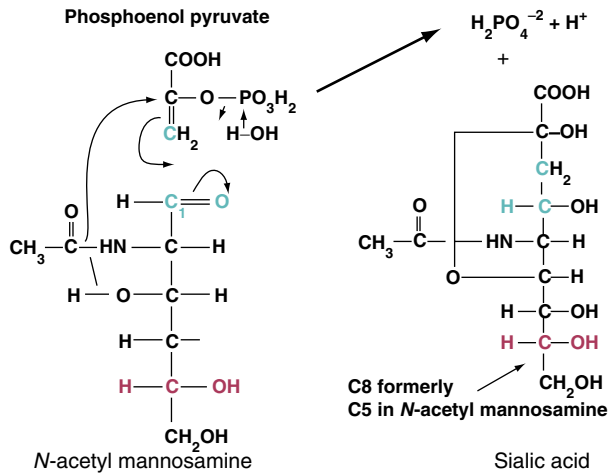


Fig. 12.5 Chemical structures of the major monosaccharides of mucins. (a) *N-Acetyl-α-galactosamine*. (b) *α-L-Fucose (6-deoxy- α-L-galactose)*. The α-anomeric bond points up in L-sugars. (c) *Sialic acid (N-Acetyl neuraminic acid)*. (a: and b: Molecular structures from <http://www.webbooks.com/MoBio/Free/Ch1B4.htm>. With permission from Web Books Publishing. Email: info2008@web-books.com; c: Molecular structures modified from an online catalogue picture published by Sigma-Aldrich, St Louis MO and reproduced with permission from Sigma-Aldrich Co. <http://www.sigmaaldrich.com/catalog/search/ProductDetail/ALDRICH/855650>)

and sialic acid (also called *N-acetyl neuraminic acid*). D-galactose is not illustrated. Fucose has a methyl group at the C6 position instead of an alcohol (-CH₂OH) or acid (-COOH) group and all hydroxyl groups attached to the ring point in the opposite direction (relative to the respective hydrogen atoms) from those in D-galactose. Within the Golgi, all O-linked glycans are made using enzymes that first recognize a nucleotide-activated monosaccharide and incorporate it at the appropriate place in a glycan chain. Fucose and sialic acid always terminate a glycan chain.

Sialic acid consists of six carbon atoms from *N-acetyl mannosamine* and three from pyruvate (Fig. 12.6) and is formed by sialic acid synthetase. This enzyme condenses the C₁ atom of mannosamine with the C₃ atom of *phosphoenolpyruvate (PEP)* in the cytosol. The 9-carbon chain product is numbered from the carboxylic acid group, the C₁ atom. The C₂ atom, the enol-phosphate of phosphoenolpyruvate becomes a keto group as in pyruvate. The C₂ ketone spontaneously forms a ketal with the OH group of C₆ (originally C₃ of *N-acetyl mannosamine*) to form a six-membered pyranose ring (right side of Fig. 12.6). The C₂ atom of sialic acid is therefore the anomeric carbon atom. The C₃ atom (originally C₃ of PEP) is connected to the C₁ atom of *N-acetyl mannosamine* (C₄ of sialic acid). The C₂ atom of mannosamine (C₅ of sialic acid) carries the *N-acetyl* group (like glucosamine and galactosamine). Sialic acid is therefore a multisubstituted pyranose ring that has only two hydroxyl groups available to form glycoside bonds, C₂ and C₄ (Fig. 12.5c).

Fig. 12.6 Sialic acid synthetase. *Right:* Reaction of the substrates, phosphoenolpyruvate and *N*-acetyl mannosamine. *Left:* Sialic acid (product). (Original Figure)



The C₂-OH group is shown as an α -anomer, because it becomes attached to a non-anomeric OH group of another glycan, most commonly the C₆ position of *N*-acetyl galactosamine in an $\alpha 2 \rightarrow 6$ bond (Fig. 12.7). Substitution at the OH group of C4 of sialic acid is never found; apparently there is no enzyme glycoside synthetase to recognize the unique configuration around the sialic acid C4 residue.

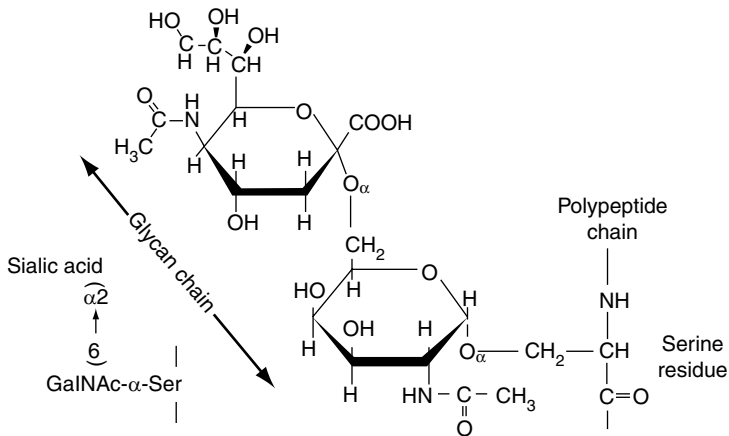


Fig. 12.7 An O-linked glycan containing sialic acid Serine (or threonine) residues are O-linked to the α -anomeric form of *N*-acetylgalactosamine (GalNAc). The anomeric -OH group of sialic acid (which is attached to C₂) becomes α -linked to the C₆-OH group of GalNAc. More complex glycans with or without sialic acid are shown in Fig. 12.8. (Original figure using structures modified from Fig. 12.5)

Salivary mucins are *N*-acetylgalactosamine linked to certain serine and threonine residues on polypeptides (O-linked) within the Golgi. The oligosaccharides possess a net negative charge from terminal sialic acid residues, or sulfate residues esterified to glycan -OH groups. The salivary mucin polypeptides, MG1 and MG2, are heavily glycosylated within an extended, flexible domain that is rich in serine, threonine, and proline. MG1 is large and forms large, disulfide bonded multimers, whereas MG2 is much smaller and forms only N-terminal head-to-head cystine-linked dimers. Mucins are only secreted by glands possessing mucous cells, although MG2 is in fact secreted by the serous cells of these glands. D-Galactose, *N*-acetyl galactosamine (GalNAc), L-fucose (6-deoxy-L-galactose), and sialic acid (*N*-acetyl neuraminic acid) are the most common sugars. Sialic acid is the most complex, possessing nine carbon atoms derived from a condensation of the C₁ aldehyde atom of acetyl mannosamine with the C₃-OH group of phosphoenolpyruvate. C₂ through C₆ form a multisubstituted six-member pyran ring in which the anomeric carbon OH group is always in the alpha orientation. Its single carboxyl group is attached to the anomeric carbon and possesses a strong net negative charge. Sialic acid and fucose are not found as intermediate sugars in an oligosaccharide chain.

12.4.1.

Mucin Glycans, ABO Antigens, and Forensic Dentistry

Mucin-type linkages (GalNAc- α 1-O-Ser/Thr) are initiated by a family of glycosyl transferases that transfer GalNAc from the sugar donor UDP-GalNAc to serine and threonine residues. Because O-linked glycosylation proceeds step-wise, addition of GalNAc to serine or threonine represents the first committed step in mucin biosynthesis. This initiating glycosyl transferase bonds the GalNAc anomeric (reducing) residue (C₁) as a α -glycoside to a recognized serine or threonine -OH group on the polypeptide. The simplest oligosaccharide (short glycan) is formed by a second enzyme bonding sialic acid to the GalNAc C₆ atom (α 2 $\rightarrow$ 6 bond; Fig. 12.7). More often, an appropriate enzyme adds a second *N*-acetylglucosamine (GlcNAc), or instead a GlcNAc residue, to the C₄ OH group of the serine or threonine-attached GalNAc, forming a (β 1 $\rightarrow$ 4 glycoside bond. Enzymes then add additional GalNAc or GlcNAc residues, or sialic acid and fucose residues to generate a diverse group of oligosaccharide structures to the central threonine/serine rich domains of MG1 and MG2.

The enzymes that synthesize these oligosaccharides also synthesize short glycans that protrude from the red blood cell surface and specify the ABO blood group antigens. In developing red blood cells, these oligosaccharides are synthesized attached to ceramide, a derivative of sphingosine that contains a saturated or monounsaturated fatty acid (Fig. 12.8).

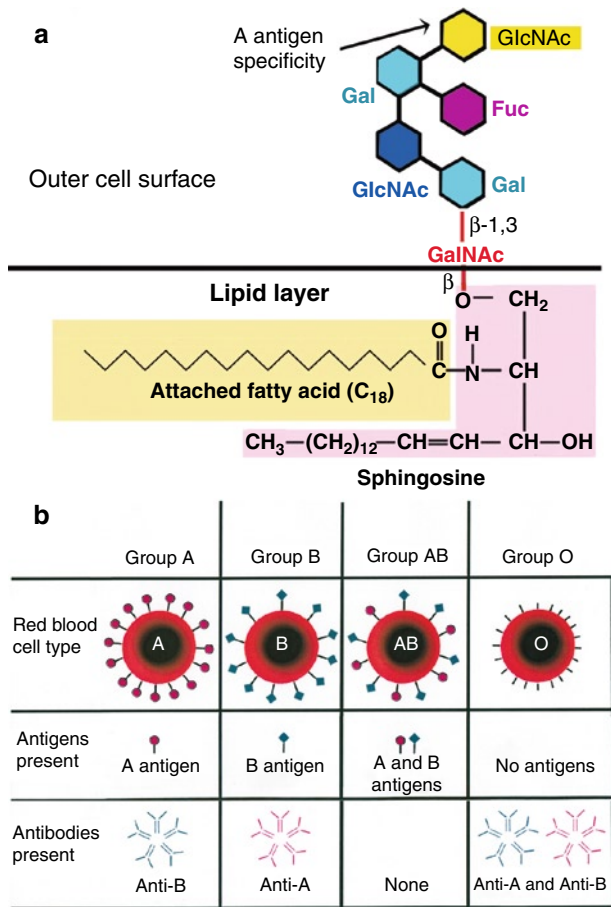


Fig. 12.8 Blood group antigen. **(a)** *Red blood cell attachment*: Ceramide, a derivative of sphingosine (purple highlight), is a common component of the outer leaflet of the cell lipid layer and possesses a free OH group. As each ceramide molecule passes through the Golgi on its way to insertion in the outer membrane of a developing red blood cell, certain enzymes recognize this structure and form the oligosaccharide (short glycan) shown. This oligosaccharide sticks out from the cell membrane and its terminal (sixth) glycan residue confers red cell antigen type. The figure shows the red cell type A specificity, in which the sixth monosaccharide is a GalNAc residue. **(b)** *ABO specificity of red blood cells*: Different enzymes add the 6th monosaccharide. GalNAc confers A antigen specificity; Gal confers B antigen specificity; both enzymes present confers AB specificity and the absence of both enzymes confers O specificity in which there are only five residues in the oligosaccharide (**a**: Figure is a pastiche of the diagram of the red blood cell A antigen in Fig. 11.17 from Berg et al., *Biochemistry*, 5th Ed. 2002, W.H. Freeman & Co., NY. Uppermost part is from Fig. 10.12 in Nelson & Cox, *Lehninger's Principles of Biochemistry*, 4th Ed. 2005, W.H. Freeman & Co., New York; **b**: Wikipedia public domain image; http://en.wikipedia.org/wiki/Image:ABO_blood_type.svg)

Ceramide is synthesized *de novo* in the endoplasmic reticulum and subsequently transported to the Golgi where it is linked to glycan as a glycosphingolipid. In the Golgi, the ceramide-OH group is recognized by the same UDP-GalNAc transferases that O-glycosylate MG1 and MG2. Following attachment, the non-reducing end of the O-linked GalNAc is attached to galactose, followed by a second GalNAc, a second galactose and then fucose (Fig. 12.8a). This sequence, GalNAc-Gal-GalNAc-Gal-Fuc is called H antigen, and it specifies the blood group O specificity (Fig. 12.9).

Some individuals have a monosaccharide transferase enzyme that adds a GalNAc residue as a second substituent to the preterminal galactose that is attached to fucose. That addition specifies the red cell A-antigen specificity (yellow highlight in Fig 12.8). Alternatively, a related enzyme adds a galactose residue (green) instead, and this addition specifies the red cell B-antigen (Fig. 12.9). Individuals who express both transferases are type AB, and each red cell has a mixture of A and B antigens (Fig. 12.8b). AB individuals

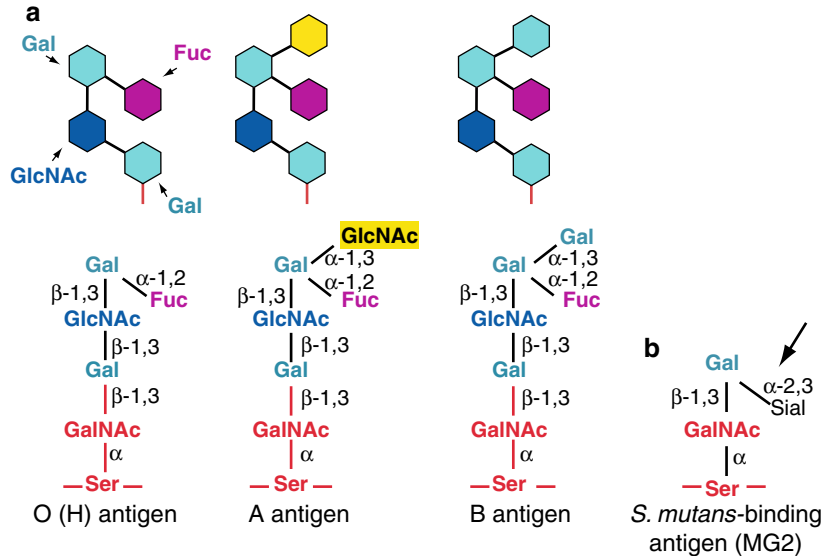


Fig. 12.9 Mucin glycan sequences. (a) *Blood group antigens*: The precursor sequence of sugars is built up on a serine or threonine -OH residue of a mucin protein and becomes O-antigen (also called H antigen) when the fifth monosaccharide, fucose, becomes attached to the 2-OH position of the previous residue, galactose [Fuc(α 1 $\rightarrow$ 2)Gal]. This sequence becomes A antigen if another enzyme (GalNAc transferase) recognizes the 3-OH group of the galactose with fucose attached and attaches a sixth monosaccharide, GalNAc (terminal yellow residue). The B antigen forms if a different enzyme (Gal transferase) attaches a galactose residue (green residue) instead. If both enzymes are expressed, an individual has 50% of glycan sequences with GalNAc and 50% with Gal (AB antigen), and if neither enzyme is present, the individual has the O antigen. (b) *Bacterial binding antigen*: In addition to blood group antigens, there are many other different glycan sequences attached to a salivary mucin serine/threonine rich domain. One such sequence is unique to the MG2 mucin and binds to *Streptococcus mutans*, an organism associated with dental caries development, Chap. 15, Sect. 2 (Modified from Fig. 11.17 in Berg. et al., Biochemistry, 5th Ed. 2002. W.H. Freeman & Co., New York)

cannot make antibodies to A or B antigen on transfused red cells because these antigens are not foreign. Only individuals lacking these antigens can make these antibodies after transfusion with donor AB cells. Donated O blood cannot induce either antibody, because the necessary terminal additions are absent. Thus, O-blood can be given to any individual in an emergency, whereas AB blood given to other than an AB individual is potentially dangerous.

The ABO glycan sequences comprise a fraction of the O-linked glycans of salivary mucins in 80% of individuals. It is not clear why the remaining 20% of individuals do not secrete these antigens. The presence of ABO antigens in salivary mucins from bite wounds in a victim who was raped or murdered is used to eliminate suspects. DNA is also present in cells that are shed into saliva and polymerase chain reaction can enhance and sequence certain fragments of the DNA from these cells as an accurate and preferred identification method, but it is expensive to perform. The presence of ABO antigens in salivary mucins often provides a rapid and relatively inexpensive initial screen to determine if a suspect can be eliminated without needing DNA testing.

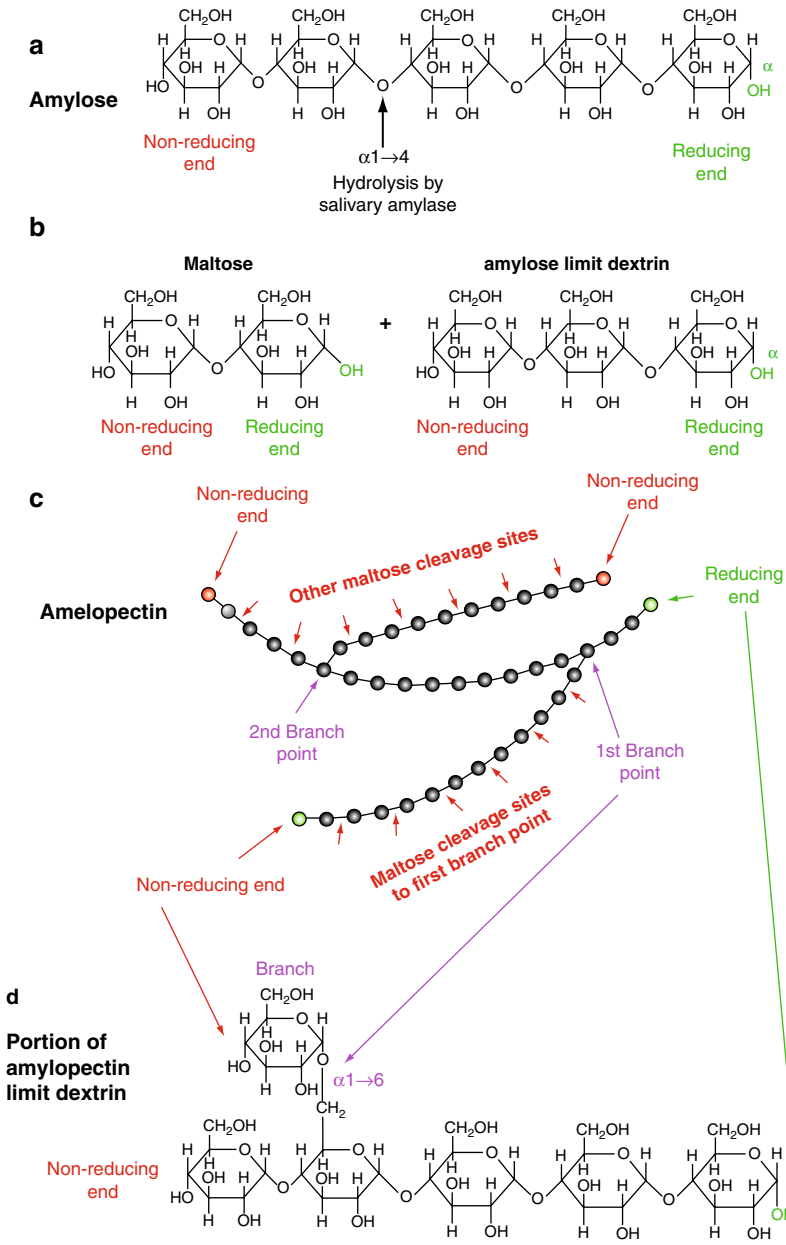
Mucin-type linkages (GalNAc- α 1-O-Ser/Thr) are initiated by a family of glycosyl transferases that transfer GalNAc from the sugar donor UDP-GalNAc to serine and threonine residues. Enzymes then add additional GalNAc or GlcNAc residues, or sialic acid and fucose residues to generate a diverse group of oligosaccharide structures to the central threonine/serine rich domains of MG1 and MG2. The same enzymes also add glycans to ceramide, a derivative of sphingosine on the outer lipid leaflet of red blood cells. These sequences stick out from the red blood cell surface and specify ABO red blood cell type. The same oligosaccharide sequences are also present attached to serine or threonine residues of salivary mucins in 80% of the population. The ABO system depends on the presence of one, other or both glycan transferases that add a terminal sugar to one of these short oligosaccharides. Individuals with one or other enzyme missing or inactive express the O antigen in which the terminal monosaccharide residue is missing. The mucin ABO specificity of saliva provides a rapid initial screen of saliva from bites on victims to exclude suspects of rape or murder.

12.5.1.

Amylase: Substrates, Products, and Mode of Action

Salivary alpha-amylase (α -amylase) is the major protein secreted by saliva and is encoded by the *Amy1* gene on chromosome 1. It is an endoglycohydrolase, which means that it hydrolyzes internal α 1 $\rightarrow$ 4 glucoside linkages. Its primary substrate is starch, which is composed exclusively of glucose in two forms, amylose and amylopectin. Amylose is a linear polymer of glucose connected by α 1 $\rightarrow$ 4 bonds (Fig. 12.10a). The α -amylase

hydrolyzes from the nonreducing end (left side in Fig. 12.10a) to give maltose ($\alpha 1 \rightarrow 4$ bonded glucose disaccharide) and a limit dextrin (Fig. 12.10b). Glucose is never a product of α -amylase digestion. Amylopectin resembles amylose but with $\alpha 1 \rightarrow 6$ cross-linked bonds (Fig. 12.10c). Amylase cuts amylopectin every two residues from the nonreducing end



end (Fig. 12.10d) to give maltose and various sized oligosaccharides, limit dextrins containing $\alpha 1 \rightarrow 4$ and $\alpha 1 \rightarrow 6$ bonds. Limit dextrins are sticky and help hold the bolus together for swallowing. Glycogen is a third substrate for amylase, but it is digested less efficiently because of its greater branching (more $\alpha 1 \rightarrow 6$ bonds).

When salivary α -amylase digests starch following a meal, it produces maltose and large limit dextrins that pass through the stomach to the small intestine. There they are further hydrolyzed by pancreatic α -amylase, which is 97% identical to salivary amylase but arises from a separate gene on chromosome 1 (*Amy2*). The limit dextrins arising from salivary and pancreatic amylase digestion pass with maltose through the mucous outer layer of intestinal epithelial cells to the brush border where α -glucosidases degrade them all to glucose. The glucose is then transported through the cells and is absorbed into the bloodstream where it raises the blood sugar levels.

The name α -amylase implies additional amylases. β -Amylase is synthesized by microorganisms in the digestive tract of animals and also by plants. It resembles salivary and pancreatic α -amylase in activity. In plants, it breaks the starch of ripening fruits into maltose to give the sweet flavor. γ -Amylases are found in a few yeasts and bacteria and is more powerful than α - or β -amylases. It hydrolyzes every second $\alpha 1 \rightarrow 4$ bond to maltose, but it also hydrolyzes $\alpha 1 \rightarrow 6$ glycoside bonds in limit dextrins to a mixture of maltose and glucose. Cellulose, a polymer of $\beta 1 \rightarrow 4$ linked glucose, is hydrolyzed by the cellulases, a set enzymes with poor homology to the amylases and absent from the human genome.

12.5.2.

Mechanism of Action of Salivary Amylase

Glycosidases such as α -amylases are very common in nature. The numerous variants expressed by different organisms show poor amino acid sequence homology, but nevertheless share a well-conserved three-dimensional structure. Moreover, this same three-dimensional structure is shared by α -glycosyl transferases such as the glucosyl- and

Fig. 12.10 Structure of starch in relation to salivary amylase action **(a)** *Amylose in starch*, Amylose is an $\alpha 1 \rightarrow 4$ polymer of glucose. A stretch of 5 glucopyranose residues from non-reducing end (red) to reducing end (green) is shown. The C_1 of glucose is the reducing end and it possesses the anomeric OH group which is shown in green and pointing down in the more common α -configuration (See Fig. 6.7 and especially Fig. 15.6). **(b)** *Amylase products of amylose*, Amylase adds a molecule of water to every second $\alpha 1 \rightarrow 4$ glycoside bond from the non-reducing end (left, red). The products are maltose (left) and a limit dextrin (right), never free glucose. The water molecule provides the OH group attached to the reducing end of maltose (green). The proton of that water molecule becomes attached to glutamate residue 233 on the enzyme (See Fig 12.11). **(c)** *Large scale amylopectin drawing showing amylase action*, Black circles indicate each glucopyranose residue. Short red arrows indicate sites of amylase hydrolysis at every second $\alpha 1 \rightarrow 4$ bond (maltose cleavage sites) beginning at the non-reducing end of each branch point. Glycogen is also a substrate, but it is more branched than amylopectin. **(d)** *Portion of an amylopectin limit dextrin*, Amylopectin has a single free anomeric OH group (one reducing end) like amylose. (Original Figures)

fructosyl-transferases of oral streptococci that metabolize sucrose and cause dental caries (Chap. 15). Homologies in amino acid sequence have resulted in these enzymes being assigned to one of three glycan hydrolase (GH) families. Family GH13 includes the mammalian α -amylases. Family GH70 includes the streptococcal α -glucosyltransferases (Chap. 15), and family GH77 includes other α -glycohydrolases and transferases.

Hydrolysis of substrates by the GH13 family begins two glucose residues from a nonreducing end. Catalysis occurs in two steps: the formation of a β -diglucosyl intermediate covalently attached to the enzyme, followed by hydrolysis of the intermediate to maltose with inversion of the β -configuration (Fig. 12.11a). These steps are reiterated

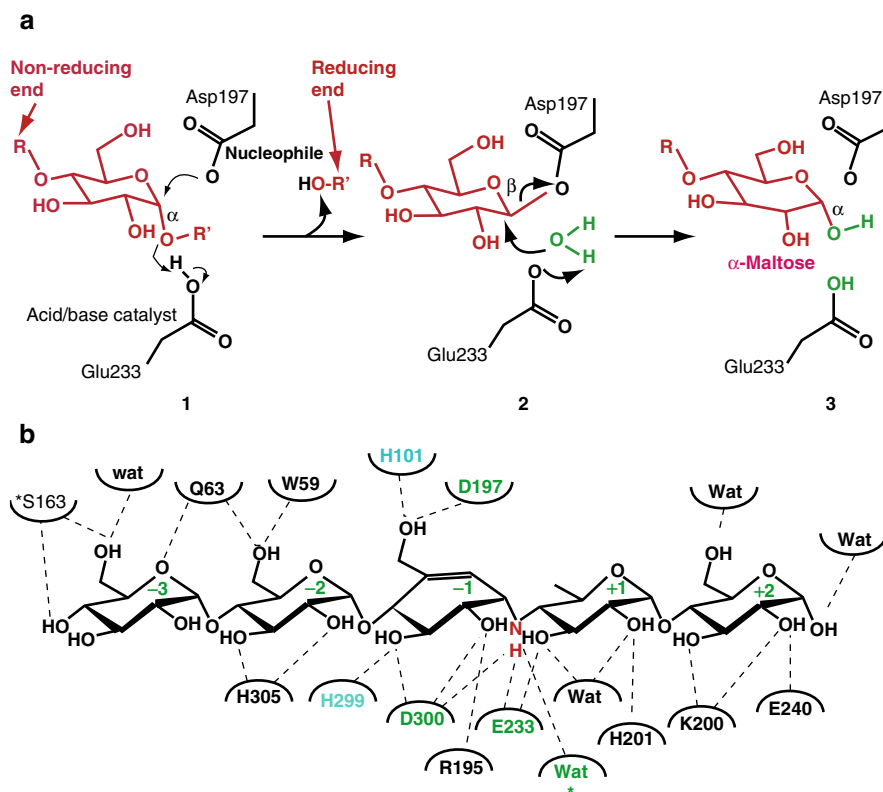


Fig. 12.11 Mechanism of salivary amylase catalysis. **(a)** Formation and breakdown of a covalent glycosyl-enzyme intermediate. Red: glucose $\alpha 1 \rightarrow 4$ polymer. Black: catalytic aspartate and glutamate residues. Green: water molecule. **(1):** Segments of at least 5–6 glucose $\alpha 1 \rightarrow 4$ residues in starch (amylose and amylopectin) bind to the enzyme such that the negatively charged carboxyl group of aspartate residue 197 attacks the glycoside bond in the center of the bound sequence, displacing its α -anomeric glycoside bond. This displacement is enhanced by a glutamate residue 233, whose side chain is nearby and accepts the electrons from the glycoside OH bond. **(2):** Asp197 and glu 233 make a nucleophilic attack on the glycoside bond two glucose residues downstream

until all possible maltose units have been removed and the residue containing the reducing end is the limit dextrin. The mechanism is facilitated by how the substrate is bound by the amino acid residues of the enzyme and the aqueous environment (Fig. 12.11b). The substrate in Fig. 12.11b is an inhibitor (acarbose) that binds like starch but contains an aminoglycoside link that cannot be hydrolyzed (red).

The enzymes of the GH13, GH70, and GH77 families all have a catalytic domain in the form of a barrel despite sequence variability. This domain was first observed in triose-phosphate isomerase, an enzyme that interconverts glyceraldehyde 3-phosphate with dihydroxyacetone 3-phosphate (Chap. 1, Fig. 2.10). The barrel is assembled from consecutive beta-strand/alpha-helix units: eight parallel beta-strands each surrounded by an alpha-helix (Fig. 12.12a). A simplified diagram of the overall structure is pictured in Fig. 12.12b. In all three glycoside hydrolase family enzymes (GH13, GH70, and GH77), the catalytic site is formed from four conserved sequences (I through IV) that come together at the top of the barrel in the tertiary structure (Fig. 12.12a; sequences numbered for salivary amylase). Sequences II and III include the catalytic aspartate or glutamate residues described in Fig. 12.11a. Sequence IV contains a second essential aspartate residue that acts with its adjacent histidine residue and the conserved histidine residue in sequence I to position the glycoside bond for hydrolysis (Fig. 12.11b). Other residues that are important for salivary amylase positioning the substrate are shown in Fig. 12.11b.

Fig. 12.11 (continued) from a nonreducing end (R). A large fragment containing the reducing end (R') is hydrolyzed and the remaining disaccharide moiety forms a covalent β -glycoside bond with asp197. **(3)**: The covalent intermediate is immediately attacked by water, restoring the α -configuration. As it does so, its proton is removed by glu 233. The cleaved disaccharide (maltose) cannot be retained on the enzyme and it falls away as a maltose residue. This mechanism applies to all α -glucosyl transferases including the bacterial glucosyl transferase that synthesizes mutan (see Sect. 15.2.1). **(b)** *Diagram of enzyme bound to the catalytic inhibitor acarbose*. This diagram is enlarged from the X-ray structure with a bound acarbose inhibitor molecule shown in Fig. 12.11a. The acarbose inhibitor is a five-membered sugar-like structure in which there is an N-linked glycosidic bond that cannot be cleaved and binds to the enzyme irreversibly. It is formed from naturally occurring acarbose by salivary or pancreatic amylase hydrolyzing and transferring residues among related structures. *Red*, glycoside amide bond of acarbose; *Green*, catalytic residues that function as shown in Fig. 12.10a; *Blue*: invariant pair of histidine residues that position the substrate correctly (see text); *Large green asterisk (bottom center)*, the catalytic water molecule; *Black asterisk (top left)* a serine residue in salivary amylase that is threonine in pancreatic amylase. Negative numbers indicate glucosyl residue binding sites on the nonreducing side of the glycoside bond that would normally be cleaved and positive numbers indicate these sites on the reducing side. When amylase or amylopectin bind instead of the acarbose inhibitor, a +3 residue site is detectable (**a**: Figure modified from Fig. 2 in Brayer GD, et al. (2000) "Subsite mapping of the human pancreatic alpha-amylase active site through structural, kinetic, and mutagenesis techniques." *Biochemistry* 39(16):4778–4791; **b**: Modified from Fig. 6 in Maurus, R. et al. (2005) "Structural and mechanistic studies of chloride induced activation of human pancreatic alpha-amylase." *Protein Science* 14(3):743–755)

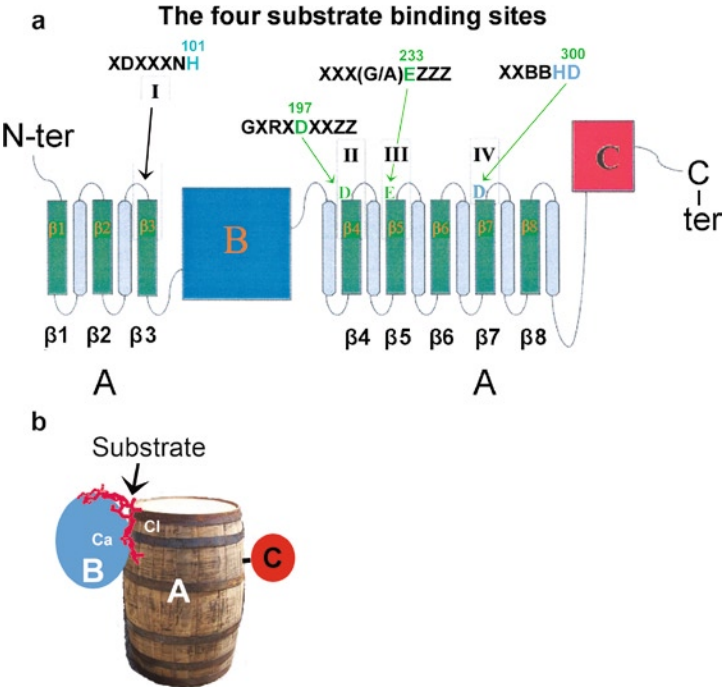


Fig. 12.12 Structure of salivary α -amylase (GH13 family). **(a)** *Topology diagram*: The secreted enzyme is a single polypeptide of 496 residues that establishes three domains, A, B, and C. The A domain consists of 8 β -sheet/ α -helical domain that forms a barrel. The β -sheets (green) are labeled β_1 through β_8 with each succeeding α -helix (light blue) not labeled. The B domain (blue) interrupts the A domain between β_3 and its corresponding α -helix (residues 100–168). The (C domain, red) is a small, structurally independent antiparallel β barrel close to the C-terminus (residues 405–496). There are four conserved regions (I–IV) of which two contain the residues essential for catalysis shown in Fig. 12.11 (Asp 197 and Glu 233; green). An additional invariant residue in conserved region I (His 101) and two in conserved region II (His 299 and Asp 300) hold the substrate so that the catalytic residues are correctly positioned (see text). In sequences I through IV, residue X = hydrophobic; B = hydrophilic; Z = amino acid sequence that determines enzyme specificity (Adapted from Fig. 1 in Kralj SE et al. (2005) “Rational transformation of *Lactobacillus reuteri* 121 reuteransucrase into a dextransucrase.” *Biochemistry* 44(25):9206–9216; sequences from Macgregor EA et al. (2001 Mar 9) “Relationship of sequence and structure to specificity in the alpha-amylase family of enzymes.” *Biochim. Biophys. Acta* 1546(1):1–20, with residue numbers altered to accord with salivary amylase) **(b)** *Diagram*: A barrel is drawn for simplicity. It is formed in the order of the β -sheet/ α -helix sequences in the polypeptide, N-terminal to C-terminal. The view is looking down from the top of a tilted (β/α)8-helix barrel (A domain) with the B domain protruding to the left. The calcium ion (red) is required for the B domain structure to interfaces correctly with the substrate binding region on the A domain at the top of the barrel (pictured on the far right). The acarbose inhibitor (substrate analogue; see legend to Fig. 12.10b) fits in a cleft between the A and B domains (red). The C-domain is loosely associated with the A domain and perhaps enhances enzyme solubility (see text)

The A domains of all mammalian and some other GH13 α -amylases have a chloride-binding site that is essential for activity together with a short, inserted downstream amino acid loop (residues 304 through 311 in salivary amylase). This loop is flexible and interacts with substrate after binding so that the catalytic residues are correctly positioned for hydrolysis. It is absent from enzymes that do not require chloride ions for activity. The C domain has no known function, but it may facilitate solubility, in part, because of glycan attached to asparagine-413 in this domain.

12.5.3. Detection of Salivary and Pancreatic Amylase

Amylase isoenzymes are detected after separation over an isoelectric pH gradient. They then diffuse into an overlying, insoluble starch polymer containing a covalently bound dye. Release of the dye indicates where the isoenzymes are on the gel. Salivary and pancreatic amylases are secreted into blood as well as saliva and small intestine and each accounts for about half the amylase content of blood plasma. More recently, monoclonal antibodies specific for human salivary amylase have facilitated the measurement of amylases in serum during salivary gland malfunction, and facilitated the differentiation of salivary from pancreatic amylases (Fig. 12.13).

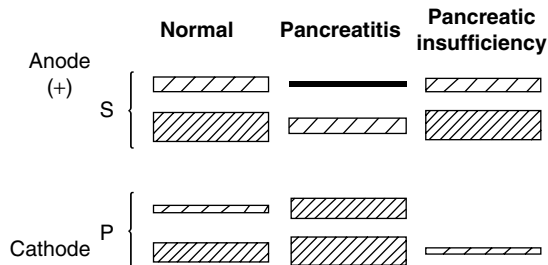


Fig. 12.13 Amylase families on electrophoresis: The appearance of multiple amylase enzymes (isoenzymes or isozymes) is due to variable loss of glycan and variable deamidation of up to three asparagine residues. Salivary amylase (S) is secreted into blood plasma as well as into the oral cavity. It is more negatively charged (has a lower pI) and therefore moves more to the anode than pancreatic amylase (P), which is also in blood plasma. The bands that move most to the anode are the most modified, but the salivary amylases are always more negatively charged (see text). In pancreatitis, pancreatic amylase isozymes are increased and in pancreatic insufficiency, the salivary isozymes are increased. Dense hatching—glycan-containing isozymes; light hatching—glycan-free isozymes (Part of Figure 1 in Pieper-Bigelow C, Strocchi A and Levitt MD (1990) “Where does serum amylase come from and where does it go?” *Gastroenterology Clinics of North America* 19:793–810. Copyright Elsevier, 1990)

In parotid and whole saliva, the glycan linked to asp-413 is often removed by enzymes in the oral cavity. In addition, this and two nearby asparagine residues may be deamidated (asn $\rightarrow$ asp) by other enzymes (deamidases). The resultant increase in net negative charge causes the appearance of amylase isozyme families after isoelectric focusing or gel electrophoresis (Fig. 12.13b). Loss of the N-terminal positive charge and deamidation of all three asparagines decrease the calculated isoelectric pH (pI) of salivary amylase from 6.34 to 5.98, similar to observed values (6.4 and 6.0). Secreted pancreatic amylase has one less negative charge and therefore migrates less to the anode. However, its observed pI is 7.0, considerably greater than its calculated pI of 6.45 (Fig. 12.13). Figure 12.13 shows also that salivary amylase is more diverse than pancreatic amylase, possibly due to cosecreted deamidases that are absent from the pancreatic secretion.

As noted previously, the N-terminal methionine residue was lost when the 15 amino acid secretory signal sequence was removed in the endoplasmic reticulum. The N-terminus of secreted salivary amylase is glutamine, which is slowly deamidated to glutamate by the deamidase that also converts the asparagine residues to aspartate as noted above. The N-terminal glutamate then slowly and spontaneously forms pyroglutamate (Fig. 12.14), which prevents amino acid sequencing by Edman degradation, interferes with protein identification by shotgun and top-down techniques, and prevents a determination of the biological functions associated with this change.

The major protein of saliva is α -amylase, an endoglycohydrolase encoded by the gene *Amy1* on chromosome 1. It hydrolyzes internal $\alpha 1 \rightarrow 4$ glucoside bonds of starch to the disaccharide maltose and moderate length oligosaccharides called limit dextrins. These products adhere to chewed food and hold the bolus together for swallowing. In the intestine, limit dextrins are digested to smaller oligosaccharides by pancreatic amylase (*Amy2*), an homologous enzyme, and then to glucose on the surface of intestinal epithelial cells. The glucose is then transported through these cells to the bloodstream. Catalysis by amylase involves an internal α -glucosyl bond becoming esterified to an asp residue on the enzyme with immediate hydrolysis by water with assistance from a nearby glu residue. The catalytic site lies at the top of a barrel-shaped domain on the enzyme. A tightly linked second domain containing Ca^{2+} stabilizes the catalytic portion of the domain and a Cl^{-1} ion within the barrel domain interacts with a short, flexible amino acid loop to position the substrate and catalytic residues correctly. Amylase is detected by isoelectric focusing followed by diffusion of the protein into an overlying, insoluble starch polymer containing a covalently bound dye. Release of the dye indicates the enzyme on the gel. Saliva and blood contain amylase isozyme families due to other enzymes removing variable amounts of attached glycan and amide groups from up to three asn residues near the C-terminus. Salivary and pancreatic amylases are also secreted into blood, where they each account for about half of the amylase content and differ by the salivary amylase family having a greater negative charge.

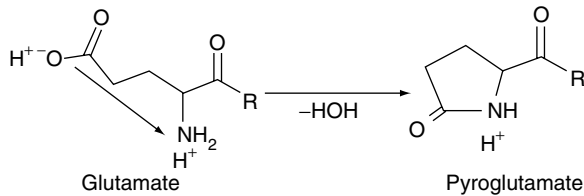


Fig. 12.14 Formation of pyroglutamate from N-terminal glutamate. The N-terminal gamma-amide of glutamine is deamidated to form glutamate (*left*). The carboxyl group of the newly formed glutamate then slowly attacks the free N-terminal amino group, removing a water molecule to form a secondary amide (see text). R is the primary amide bond of the alpha-carboxyl group of glutamine (or glutamate) to the alpha amino group of the next amino acid of the primary sequence (tyrosine) (Slightly modified from Fig. 1 of Chelius D, et al. (2006) "Formation of Pyroglutamic Acid from N-terminal glutamic acid in immunoglobulin gamma antibodies." *Anal. Chem.* 78(7): 2370–2376)

12.6.1.

Proline-Rich Proteins

About 40% of the non-mucous proteins in salivary secretions are proline-rich proteins (PRPs), which are about equally divided into, acidic, basic, and glycosylated proteins. The N-terminal domain of the acidic and basic PRPs is dominated by either negatively or positively charged amino acids and their C-terminal domain by many proline residues. This simple 2-domain primary structure, in which each half has a predominance of specific amino acids, is diagrammed in Fig. 12.15. The acidic PRPs are rich in aspartate and glutamate residues at the N-terminal domain and are encoded by two genes, *PRH1* and *PRH2*. The basic PRPs are rich in lysine, arginine, and histidine residues at their N-terminal domain and encoded by four genes, *PRB1*, 2, 3, and 4. Each gene has several alleles that vary in size and their products account for about 20 proteins, giving rise to complex polymorphic patterns between individuals. During and after secretion, various proteins arise by partial proteolytic cleavage at sites with sequence arg-pro-pro-arg in acidic PRPs and arg-ser-X-arg in basic PRPs (where X stands for any amino acid). The glycosylated PRPs are all basic and the glycan is asparagine-linked as in amylase.

As noted earlier (Sect. 12.1.3), the reduced enamel epithelium is replaced with adherent salivary proteins, mostly amylase and acidic proline-rich proteins. The acidic PRPs are at about a three times greater concentration than in secreted saliva. The acidic domain of these proteins especially has a high affinity for hydroxyapatite. Once bound to pellicle, the N-terminus of acidic-PRPs forms an attachment site for the major classes of commensal bacteria (viridans streptococci and *Actinomyces* spp.) that occupy the mucosal surfaces and saliva of a healthy oral cavity. Some streptococci grow by themselves in dental biofilms, but many others grow better if certain *Actinomyces* spp. are also present.

many oral streptococci. All bacterial binding to salivary agglutinin is believed to occur at a single site in SRCR domain 2 near the N-terminus (Fig. 12.16). Salivary agglutinin remains soluble after binding bacteria, and it is part of the innate immunity system that clears bacteria from the oral cavity (Table 12.1 and Sect. 12.1.4). Use of a monoclonal antibody to quantify the amounts of salivary agglutinin have shown that it is present in small but highly variable amounts. Because salivary agglutinin contributes also to acquired pellicle, individuals who secrete more agglutinin may bind a greater complexity of bacteria to teeth surfaces. Indeed, the presence of certain acidic PRPs along with a greater amounts of salivary agglutinin increase an adult's susceptibility to caries (Sect. 15.3.3).

Salivary agglutinin is a member of the *scavenger receptor cysteine-rich (SRCR)* superfamily, a group of large (~2,400 amino acids; mol wt 262 kDa) cell surface and secreted glycoproteins. These glycoproteins are characterized by SRCR and SRCR-interspersed (SID) domains illustrated in Fig. 12.16. Other proteins in this family include: (1) the

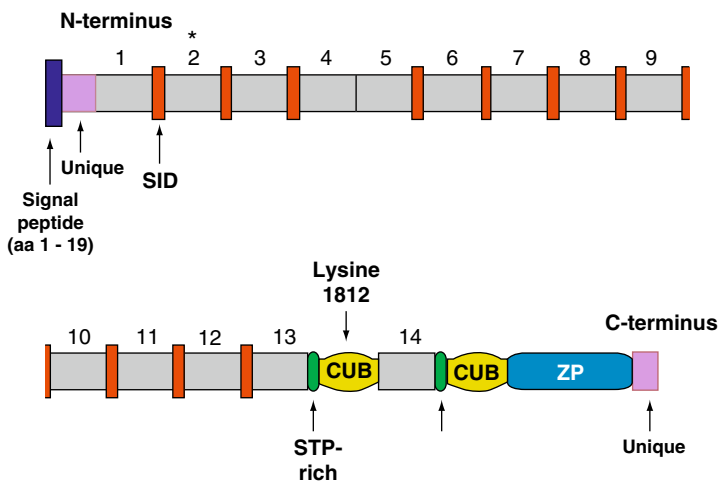


Fig. 12.16 Salivary agglutinin. Salivary agglutinin is characterized by 14 SRCR domains (gray and numbered) and many SRCR-interspersed-domains (SID). SRCR and SID domains are ~100 and ~30 amino acids in length, respectively, and the secreted molecule consists of a total of 2,394 amino acids. SRCR domains are described in the text. The N-terminal methionine and following 19 amino acids (signal sequence, black) is cleaved in the endoplasmic reticulum. The N-terminal domain of the secreted protein (purple) has a short unique domain before the first SRCR domain. The asterisk above SRCR domain 2 indicates a unique bacterial binding site (see text). Like the N-terminal domain of the secreted protein, the C-terminal domain (purple) is also short and unique. Immediately upstream of the C-terminus is a zona pellucida (ZP) domain (blue) and 2 CUB domains (yellow, see Chap. 8) before and after the 14th SRCR domain. Immediately upstream of each CUB domain is a short serine-threonine-proline-rich (STP-rich) domain (green). The ZP domain consists of 260 amino acids just upstream of the C-terminus and is common in membrane-anchored eukaryotic glycoproteins possessing a large multidomain, extracellular region. However, salivary agglutinin is secreted, not membrane anchored. The STP-rich domain consists of 25 amino acids of which 19 are serine, threonine, or proline residues forming five possible O-linked glycosylation sites. (Figure is simplified from Bikker et al. (2002 Aug 30) "Identification of the bacteria-binding peptide domain on salivary agglutinin (gp-340/DMBT1), a member of the scavenger receptor cysteine-rich superfamily." J. Biol. Chem. 277(35):32109–32115)

macrophage scavenger receptor involved in the endocytosis of low-density lipoproteins by macrophages; (2) human macrophage-associated galectin3-binding protein also called Mac-2 (a lectin is a carbohydrate binding protein and a galectin is a protein that binds to β -galactosides); and (3) cell differentiation (CD) antigens CD5 and CD6 involved in the activation and differentiation of thymus-derived lymphocytes in response to foreign substances (antigens).

The salivary proline-rich proteins have a two-domain structure, a proline poor N-terminal domain that is acidic or basic and determines enamel binding, and a proline-rich C-terminal domain that determines bacterial binding. Individual variations in acidic proline-rich proline allelic composition and in the amount of salivary agglutinin, a secreted innate immunity protein that binds bacteria, may account for differences in biofilm composition and dental caries susceptibility.

Periodontal disease is a mixture of diseases in which the periodontal attachment is destroyed, resulting in loose teeth that may exfoliate. This chapter describes how chronic periodontitis, the common form of this disease, begins as gingivitis and is induced by bacteria (Sect. 1), how in turn the bacteria induce the neutrophil infiltrates responsible for gingivitis (Sect. 2), and how the inflammatory response becomes destructive with macrophage activation (Sect. 3), concluding with a discussion of mechanisms of cell death in chronic periodontitis (Sect. 4). The last section discusses the roles of eicosanoids in promoting and resolving periodontal inflammation, and the effects of nonsteroidal antiinflammatory drugs on these processes (Sect. 5).

13.1.1 Detecting Periodontal Disease

Periodontal disease describes a mixture of diseases in which the periodontal attachment is destroyed, resulting in loose teeth that may exfoliate. Periodontitis is divided into chronic and aggressive forms that are localized or generalized (affect few or many teeth). Chronic periodontitis is very common (Sects. 13.1.2–13.4.3), whereas aggressive periodontitis is rare (Chap. 14). The collagen fibers of the gingiva and periodontium are described in Chap. 3 (Sect. 3.1.5), and its epithelial cover in Chap. 5 (Sect. 5.2.3).

Chronic periodontitis first appears at the gingival sulcus, the tooth-soft tissue interface (Fig. 13.1a). The marginal gingiva becomes red, swells with loss of collagen, and bleeds on gentle probing (gingival inflammation or gingivitis, Fig. 13.1b). If the gingivitis and collagen loss extends into the underlying periodontium, periodontitis is present (Fig. 13.1c). Gingival inflammation is more prominent in chronic than aggressive periodontitis. The severity of periodontitis is measured by periodontal attachment loss: distance in millimeters from the enamel–cemental junction to the base of the gingival sulcus or pocket (deepened sulcus). Measurements are made at six sites on each tooth: at mesial, central, and distal surfaces of the buccal and lingual sides. A mean attachment loss of up to 2 mm indicates mild periodontitis; 2–4 mm indicates moderate periodontitis; and more than 4 mm advanced periodontitis.

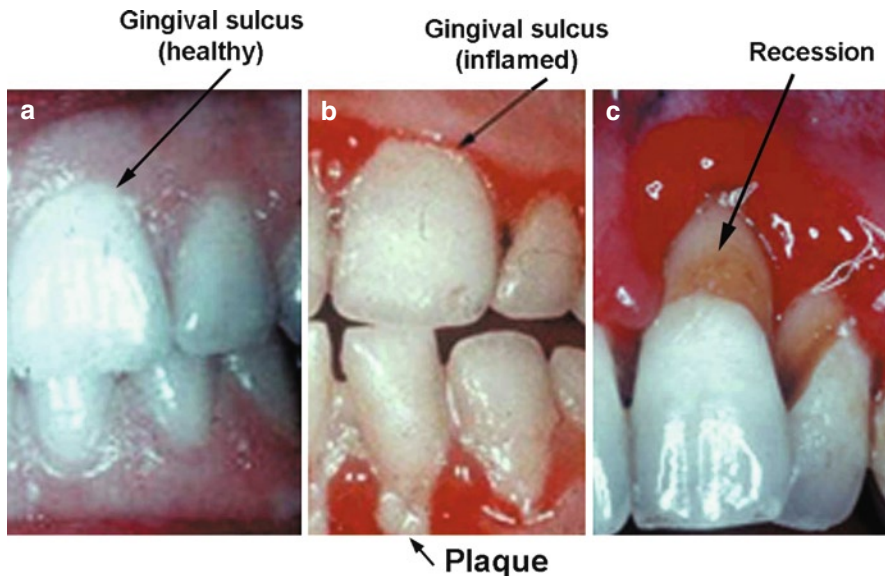


Fig. 13.1 Healthy gingiva, gingivitis, and periodontitis in the oral cavity. **(a)** *Healthy gingiva*. The pale-pink, stippled gingiva is attached tightly to clean teeth. The gingival sulcus is indicated (arrow). **(b)** *Gingivitis*. The gingiva is red, especially at the free gingival margins (upper arrow) and plaque is present (lower arrow). Patients with moderate to severe gingivitis (illustrated in this slide) complain that their gums bleed on toothbrushing. **(c)** *Periodontitis*: The gingiva has receded and the cementum has been abraided, exposing the dentinal surface of the tooth root (recession, arrow). The gingiva is inflamed (red) and the sulcus is deepened. Periodontal pockets have formed between the exposed dentin and the inflamed (red) gingival

13.1.2

Gingivitis and Chronic Periodontitis in Humans and Animals

The present-day understanding of how the commensal microbiota causes gingivitis began with a human study of experimental gingivitis by Harald L  e and his colleagues in Denmark in 1965. Adults with no periodontal attachment loss are selected, and their teeth are thoroughly cleaned for a few days. They then abstain from oral hygiene procedures for 3 weeks. A microbial biofilm (plaque) adheres to teeth, and an inflammatory exudate appears at the sulcus, the *Gingival Crevicular Fluid* (GCF; Table 13.1). This fluid is derived from serum, *blood plasma in which fibrin clot formation is prevented by plasmin activation* (Sect. 13.2.3). After a week, gingivitis appears and extends to all teeth within about 3 weeks. The severity of gingivitis is measured by gingival index (Tables 13.2) averaged across the sites described above for attachment level. Dogs develop periodontal disease as in humans, except that a hard diet cleans the teeth instead of oral hygiene. Changing dogs from hard to soft diets causes biofilm accumulation and gingival inflammation, which progress to periodontitis. Other animal models are less satisfactory. Gingivitis and periodontitis are induced in non-human primates only if silk ligatures are placed at the gingival margins. In rodents, periodontitis only develops if the endogenous commensal microbiota is depressed by antibiotics and the oral cavity monoinfected

Table 13.1 Interstitial and gingival crevicular fluid composition

ISF	GCF
Transudate	Exudate
No proteins	Rich in proteins
No neutrophils	Many neutrophils

Table 13.2 The gingival index

Marginal gingiva	Score
Pink and stippled ^a	0
Red and edematous ^b	1
Bleeds ^c within 10 s	2
Bleeds ^c immediately	3

^aFig. 13.1a

^bFig. 13.1b

^cAfter gentle probing

with an oral bacterial species. Hair and bedding become trapped in gingival pockets and may enhance periodontitis development. Gingival inflammation is minimal.

13.1.3

Microbiota of Gingivitis and Chronic Periodontitis in Man

Because animals require sedation before sampling the oral cavity, most studies have been of the human oral microbiota. These studies indicate that, in a healthy oral cavity, bacteria attach to the oral mucosa and are continually shed into saliva (Sect. 12.1.5). This *commensal microbiota* is present in saliva (Sect 12.1.5) and loosely adherent to the oral mucosa. It is mostly composed of viridans streptococci and *Actinomyces* species (Fig. 13.2a, b). These bacteria hydrolyze sialic acid and glycans from mucins and glycoproteins on the mucosal surface or in saliva (Sect. 12.3.1), and they grow by metabolizing the glycans to carbon dioxide and water (respiration, Sect. 1.3.1). They also attach to teeth surfaces where *mutualistic interactions*, characterized by luxuriant mixed culture growth on saliva alone *in vitro* or *in vivo*, generate a biofilm. These interactions are called *quorum sensing*, and they are mediated by 4,5-dihydroxy-2,3-pentanedione (DPD; Fig. 13.3a).

DPD is also called *autoinducer-2* (AI-2; AI-1 is another, less common inducer). AI-2 is derived from S-ribosyl homocysteine which is hydrolyzed to homocysteine and 1-deoxy-3-dehydro-D-ribulose in bacteria. The D-ribulose derivative is extruded as a boric acid ester (boron is a trace element, Chap. 1), which spontaneously rearranges into numerous isomers that, together, comprise AI-2. Each isomer binds with a structurally and functionally distinct receptor. Thus, quorum sensing through AI-2 secretion activates the expression of genes required for growth by mutualistic interactions within biofilms. For example, coaggregation with an actinomyces species enables a streptococcus species to switch from quiescence to active growth when arginine is scarce as it is in saliva.

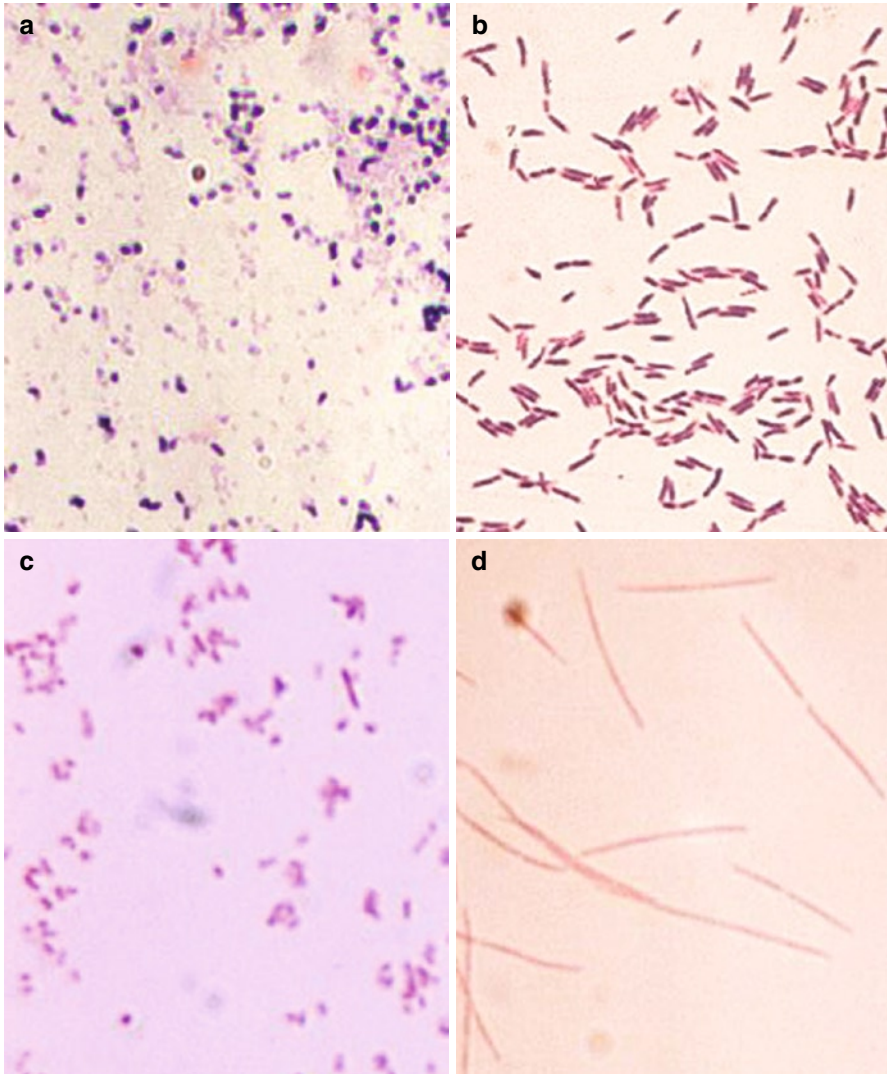


Fig. 13.2 Common bacteria of plaque. Slides show typical gram stain for: (a) viridans streptococci; (b) *Actinomyces naeslundii*, previously *A. viscosus*; (c) *Eikenella corrodens*, and (d) *Fusobacterium nucleatum* (From Public Health Image Library (PHIL) at the CDC – Bacteria Site: <http://phil.cdc.gov/phil/home.asp>)

Another bacterium that contributes to commensal biofilms is *Eikenella corrodens*, a gram negative, small rod (Fig. 13.2c). *E. corrodens* grows in a healthy oral cavity by reducing nitrate in saliva to nitrite (Sects. 1.3.2 and 12.1.3). It is an important contributor to bite wound infections and is also the major known producer of lysine decarboxylase, which converts lysine to cadaverine and carbon dioxide (Fig. 13.4). Lysine is a nutritionally essential amino acid, whereas cadaverine is not. In humans, *E. corrodens* and lysine

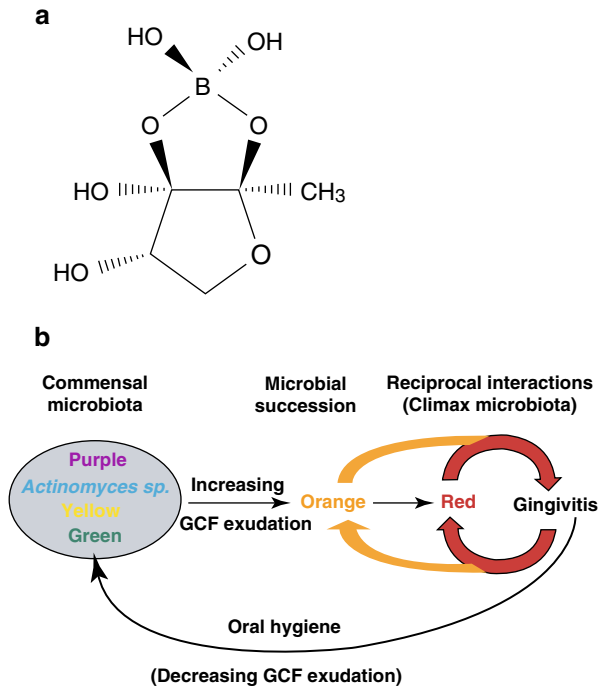


Fig. 13.3 Bacterial colonization and succession in gingivitis. (a) *Structure of autoinducer-2*. All possible configurations of the OH groups occur spontaneously in solution due to boron-catalyzed hydrolysis and rearrangement. Different bacterial species have receptors that only recognize one or a limited number of these configurations (b) *Bacterial complexes*. The commensal bacteria attach to teeth as distinct complexes within a biofilm (plaque). The most prominent complexes are *Actinomyces* spp. (blue), *Streptococcus* spp. (yellow) and a mixture of *Capnocytophaga* spp. with *E. corrodens* (green). The successor microbiota attaches to these commensal biofilms as complexes of *Fusobacteria* spp. with many other gram negative bacteria (orange) and a climax complex of just three species: *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* (red) (a: Public domain image: <http://en.wikipedia.org/wiki/Autoinducer-2>; b: Modified from Fig. 1. in Socransky and Haffajee, "Periodontal microbial ecology." Periodontology 2000, 38:135–187, Blackwell Munksgaard 2005)

decarboxylase activity increase markedly in teeth-adherent biofilms during the first week of experimental human gingivitis.

Lysine decarboxylase may play a critical role in initiating gingivitis because basal cells of the epithelial attachment at the base of gingival sulci must proliferate continuously to maintain a basal lamina seal (Sect. 5.2.3). By starving these epithelial cells of lysine, their proliferation and ability to seal the base of a sulcus are impaired. This means that commensal microbial products could better access the gingiva where they induce inflammation and GCF exudation (Sect. 13.2.1). *E. corrodens* is a common cause of dog bite infections, and if dogs are fed a soft diet to accumulate biofilm faster (Sect. 13.1.2), immunization with purified lysine decarboxylase could induce antibodies that slow gingivitis development. The GCF is a richer substrate for bacterial growth than saliva, and it promotes the development of a predominantly gram negative *successor* and *climax* microbiota in

gingival sulci (Fig. 13.3b). Individual bacteria contributing to the successor microbiota constantly enter the oral cavity from the environment. Some coaggregate within the biofilms and generate autoinducer-2 levels that promote their growth in GCF at the expense of the commensal microbiota. *It is generally accepted that the successor microbiota are mostly responsible for gingivitis and chronic periodontitis.*

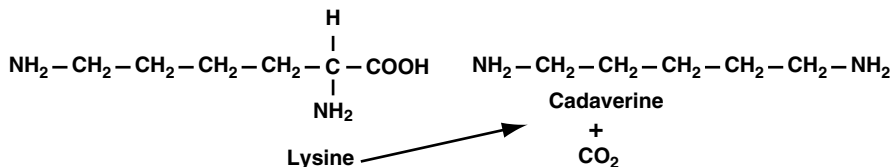


Fig. 13.4 Reaction catalyzed by lysine decarboxylase

The metabolism of the successor microbiota is asaccharolytic. Its various bacteria release ammonia from all amino acids and sulfides that cause oral malodor from cysteine and methionine amino acids (Sect. 1.3.2). When albumin, citrate, and pyrophosphate in the GCF are hydrolyzed, calcium ions (Sect. 9.1.4) become free to precipitate with phosphate ions and form calculus. Calculus protects the biofilm at the base of a gingival sulcus, promoting persistent inflammation that impairs the viability of the junctional epithelial attachment (Sect. 13.4.1). Salivary gland loss or malfunction, or persistent mouth-breathing or tobacco smoking, dry up the mouth and enhance bacterial colonization and disease (Section 12.1.3). In periodontitis, bacterial products pass from gingival capillaries into the systemic circulation and promote inflammation that worsens some preexisting diseases, notably cardiovascular disease, and adult-onset diabetes. Periodontitis also predisposes to bacterial endocarditis (bacteria attaching to heart valves) and low birth-weight babies.

13.1.4 Drugs to Prevent Gingivitis

In addition to toothbrushes and dental floss for mechanical oral hygiene (Sect. 13.5.1), antiseptic mouthwashes exert chemical control of the microbiota. The most commonly sold mouthwashes in the United States are made from mixtures of volatile aromatic compounds from plants. Different mixtures of these plant compounds possess a unique scent or *essence* and are commonly called “*essential oil*” mixtures. They have antiseptic properties that control the successor microbiota, but a need for 15–20% alcohol to maintain “*essential oil*” solubility has been linked to an increased risk of oral cancer in long-time daily human users. Less popular mouthwashes contain an antiseptic called chlorhexidine (Peridex) or a detergent that interferes with bacterial biofilm aggregation called delpolminol (Decapinol). Long-term use of all of these mouthwashes interferes with taste and *probiotic*, protection by the commensal microbiota from pathogenic organisms.

The next generation of drugs to supplement oral hygiene may interfere more specifically with one of four actions: (a) initial stages of bacterial attachment to teeth surfaces; (b) coaggregation of successor bacteria into a biofilm; (c) quorum sensing; and (d) lysine

decarboxylase impairment of the epithelial attachment barrier. Recent studies by the author suggest that immunological inhibition of *E. corrodens* lysine decarboxylase in beagle dogs, or chemical inhibition of this enzyme in humans, retards gingivitis development and therefore the eventual development of chronic periodontitis.

Periodontal disease describes a mixture of diseases in which periodontal attachment is destroyed. It may be chronic or aggressive, and localized or generalized. Chronic periodontitis first appears as gingival inflammation (gingivitis) accompanied by a teeth adherent microbial biofilm (plaque) and calculus. Bacteria are always present in the oral cavity. They grow on mucosal surfaces, in saliva, and adhere to teeth as biofilms within which quorum sensing generates chemicals that activate mutualistic interactions leading to luxuriant mixed culture growth. *Eikenella corrodens* in the commensal microbiota is a major source of lysine decarboxylase, which depletes the interstitial fluid of lysine necessary to maintain an intact epithelial barrier at the base of gingival sulci. Loss of this barrier likely increases exposure of the gingiva to individual members of a gram negative, successor microbiota that induce an inflammatory exudate derived from blood plasma, the gingival crevicular fluid, which is richer in bacterial substrates than saliva. The GCF promotes quorum sensing by the successor microbiota at the expense of the commensal microbiota and gingivitis appears. Current mouthwashes contain antiseptics or detergents that supplement oral hygiene but interfere with probiosis, protection of the oral cavity by the commensal microbiota. New drugs that maintain probiosis would inhibit bacterial attachment to teeth, quorum sensing, or lysine decarboxylase impairment of the epithelial barrier.

13.2.1

Mammalian Cells Recognize Prokaryotic Molecules

The successor microbiota is a major source of products containing conserved prokaryotic motifs, *pathogen-associated molecular patterns* (PAMPs). The best-known PAMPs are *lipopolysaccharides* of gram negative bacteria (Sect. 1.4.2). Lipopolysaccharides are recognized by *pattern-recognition receptors* (PRRs) known as *toll-like receptors* (TLRs) on the mammalian cell surface. *[TLRs received their name from their homology to a protein encoded by a gene identified in 1985 from Drosophila flies. Mutations of this gene made the flies look so unusual, that German researchers spontaneously called out “toll,” their word for “amazing.”]* Other PAMPs are membrane permeable, for example, bacterial peptidoglycan fragments (Sect. 1.4.1), fatty acids hydrolyzed from lipopolysaccharides (Sect. 1.4.2), bacterial DNA fragments, and various other products of lysed bacteria. These agents are recognized by PRR proteins that are soluble in the cytosol and have an amino acid sequence homology to *nucleotide-binding oligomerization domain* (NOD) proteins. There are 23 such *NOD-like receptor proteins* (NLRs) in humans, some of which have alternative

names derived from findings before this family and its functions were recognized. PAMP-bound TLRs mediate inflammation, whereas PAMP-bound NLRs mediate either inflammation or tissue destruction, depending on the state of the cell and type of molecule recognized (Sect. 13.4.1).

13.2.2

PAMPs Induce PRRs to Release Cytokines That Attract Leukocytes

Innate immunity to infections is mediated by many of the same enzymes that function in bone demineralization, i.e., acid-activated lysosomal enzymes (Sect. 10.1.1). These enzymes are brought to infected regions by leukocytes (neutrophils and macrophages). PAMP-bound PRRs release two endogenous ligands (proinflammatory cytokines) that attract and activate leukocytes from the cytosol of junctional epithelial cells and gingival fibroblasts. One important cytokine, *interleukin-1* (IL-1), is released from the cell cytosol by ligand-bound PRRs causing changes in membrane associated proteins without involving the Golgi or secretory pathways.

IL-1 is stable, and it diffuses to and activates receptors (IL-1 receptors) on adjacent and distant cells. The IL-1 receptor of endothelial cells is especially important (Fig. 13.5a). In all cells, the IL-1 ligand bound to its receptor causes surface expression of a second cytokine, *tumor necrosis factor- α* (TNF- α). *TNF- α was originally described as a factor that kills (causes necrosis of) mouse fibrosarcoma (cancer) cells but not normal mouse fibroblasts.* TNF- α is released from the cell surface by an adamalysin protease, *TNF alpha converting enzyme* (TACE). TACE is also known as ADAM17, one of more than 40 cell surface bound adamalysins related to the ADAM-TS2 subfamily (Sect. 8.2.1). Unlike IL-1, TNF- α is unstable and can only activate TNF receptors on nearby cells. It usually produces responses that supplement those from IL-1 activation (Fig. 13.5b).

IL-1 is released as two forms, IL-1 α and IL-1 β , encoded by separate genes that are only 28% homologous. The expressed polypeptides form a 12-stranded β -sheet around a central axis such that two sets of six β -sheets come together to form an antiparallel β -barrel (Fig. 13.6a). Similar β -barrel structures are present in α -amylase and many other proteins that recognize carbohydrates (Sect. 12.5.2). IL-1 is guided to its receptor by interacting first with glycans on the cell surface. The receptor-binding domain, the C-terminal domain (Fig. 13.6b), is correctly folded in IL-1 α , but that of IL-1 β must be cleaved from its N-terminal domain by *interleukin-1 converting enzyme* (ICE). This calcium ion-activated serine protease, also called *caspase-1* (Sect. 13.4.1), is secreted by fibroblasts and macrophages. The active form of IL-1 β is therefore one third smaller than IL-1 α . Other proteases secreted by the successor microbiota, for example, *T. denticola* in the climax microbial complex (Fig. 13.3), also activate IL-1 β . Keratinocytes, including junctional epithelial basal cells exposed to PAMPs, secrete IL-1 β along with IL-1 α , whereas fibroblasts and macrophages secrete IL-1 β and ICE.

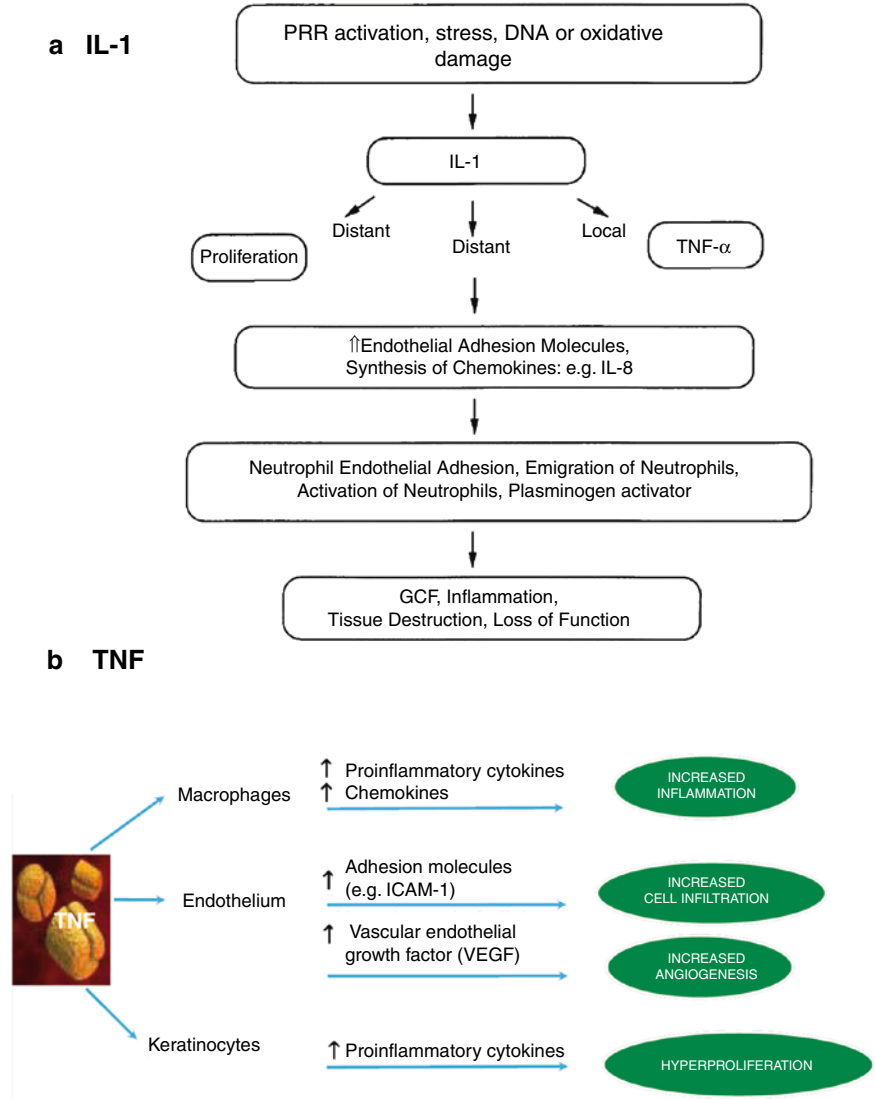


Fig. 13.5 How IL-1 and TNF- α cause inflammation. **(a) IL-1 function.** IL-1 induces the secretion of TNF- α to enhance its actions. The major effect of IL-1 is on capillary endothelial cells (see text). IL-1 causes keratinocytes and endothelial cells to proliferate and secrete other interleukins, especially IL-8, which acts like IL-1 on capillary endothelial cells **(b) TNF- α function.** TNF- α usually acts as a proinflammatory growth and survival factor (see text). Its induction of vascular endothelial growth factor (VEGF) promotes greater capillary proliferation and permeability. VEGF contains a cysteine knot domain like von Willebrand factor (Fig. 11.2) [a: Based on Fig. 1 in Dinarello CA (2000) "Proinflammatory Cytokines." Chest 118(2):503–508; b: Slightly modified Fig. 1 from Jackson JM (2007); TNF- α inhibitors, Dermatologic Therapy, 20:251–261]

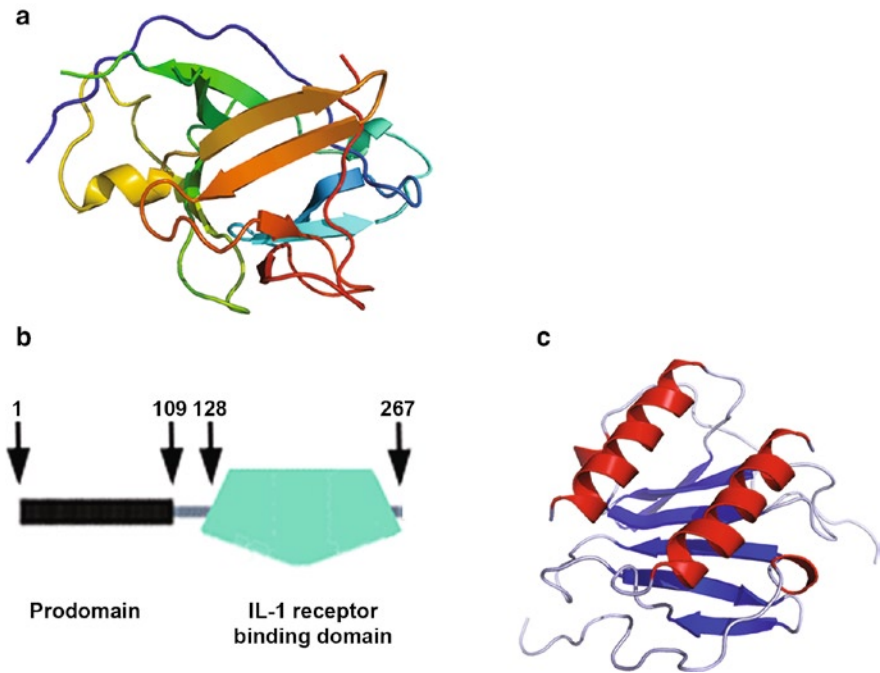


Fig. 13.6 Structures of IL-1 α and IL-8. **(a)** *Structures of Interleukin-1*. Interleukins are a family of intercellular signaling molecules (cytokines) that are ligands for a specific receptor, the IL-1 receptor. IL-1 forms a 12-stranded β -sheet structure, several regions of which, but especially a loop between strands 4 and 5, are implicated in binding to the IL-1 receptor. **(b)** *Domain structure of IL-1*. IL-1 α is composed of an N-terminal prodomain (residues 1–109), a short unfolded connector region (residues 110–128) and a large C-terminal domain (residues 129–267) containing the β -sheet conformation shown in **(a)** above. IL-1 β is similarly constructed, but two residues shorter than IL-1 α . IL-1 α is active without cleavage, whereas the prodomain of IL-1 β , which ends at residue 103, prevents receptor-binding unless it is cleaved in the connector domain (residues 104 – 118) C-terminal to aspartate residue 116 (see text). The large fragment contains a homologous β -sheet domain (residues 119–265). **(c)** *Interleukin-8 (IL-8)*. IL-8 is not of the interleukin family. It is one of a family of 13 CXC chemokines, small, positively charged proteins that attract and activate leukocytes (see text). The many chemokines in the body are divided into CXC and CC subfamilies, based on the arrangement of the first two of four conserved cysteine residues; the two cysteines are separated by a single amino acid in CXC chemokines but lie next to each other in CC chemokines. Chemokine structures all have a double α -helix that interacts with the receptor following heparin binding (**a**: Figure is public domain; <http://en.wikipedia.org/wiki/Image:2ILA.png>; **b**: Figure is modified from an EMBO SMART diagram of IL-1 at http://smart.embl.de/smart/show_motifs.pl?ID=P01583; **c**: Figure constructed by Ramin Herati, 12-10- 2006 as a Wikipedia public domain image http://en.wikipedia.org/wiki/Image:IL8_Solution_Structure.rsh.png)

Ligand-bound IL-1 and TNF receptors activate intracellular protein kinases called *mitogen-activated protein* (MAP) kinases. These kinases phosphorylate serine or threonine residues on adjacent proteins in response to extracellular stimuli that were first identified as mitogenic because they induced fibroblast growth factors (Sect. 13.2.5). IL-1 or

TNF receptor activation of these kinases stimulates NF κ B, a transcription factor that induces the expression of proteins responsible for inflammation and demineralization (Sect. 10.2.2), not growth. Expressed NF κ B gene products cause the IL-1 and TNF- α activities listed in Fig. 13.5.

13.2.3

IL-1: A Host Mediator of Gingival Inflammation

In gingivitis, IL-1 production by PAMP activation of external and cytosolic PRRs is maximal within the junctional epithelium. As little as 0.1 ng/mL of IL-1 will bind to and activate IL-1 receptors in capillary endothelium where they: (a) Open intercellular junctions allowing plasma to exude; (b) Promote secretion of plasminogen activator to prevent the plasma fluid exudate from clotting in the stroma (Sect. 11.4.2); (c) Induce *intercellular adhesion molecule-1* (ICAM-1) to arrest and transfer neutrophils from blood to the exudate; and D) Induce TNF- α to enhance ICAM-1 and VEGF production.

Neutrophils are slowed within the blood flow by possessing a receptor that binds to an endothelial protein protruding into the capillary lumen (*E-selectin*). The ligand-receptor complex activates cytosolic proteins to transmit a signal that extends a β_2 integrin, *Lymphocyte function-associated antigen-1* (LFA-1) by inside-out signaling (Sect. 4.4.1). The extended LFA-1 binds to the ICAM-1 induced by IL-1 and the double receptor-ligand binding (Fig. 13.7) arrests neutrophils in the blood flow. It also activates a respiratory burst that provides energy for the neutrophils to migrate through the opened intercellular junctions with a fluid exudate that becomes the GCF.

IL-1 also induces epithelial and endothelial cells to secrete *IL-8*, a chemokine misnamed an interleukin (Fig. 13.6c), and neutrophils to secrete leukotriene B₄, an eicosanoid (Sects. 13.5.2 and 13.5.3). IL-8 and leukotriene B₄ enhance ICAM-1 expression from the capillary endothelium, cause neutrophils to emigrate and accumulate on the outer surface of the opened capillaries, and migrate with the GCF through the junctional epithelium where IL-1 production is greatest. The expanded, leaky capillary plexus in the stroma beneath the mucosal covering of the gingival sulcus (section 3.3.1) swells the gingiva and may cause spontaneous sulcular bleeding on probing.

13.2.4

Neutrophils Function in Tissue Destruction

Neutrophils contain secretory vesicles and granules. The former deliver their contents during passage through the capillary wall, whereas granule secretion is activated in the stroma by a β_2 integrin that binds to type I collagen and other stromal ligands. This promiscuous receptor, $\alpha_m\beta_2$ (Fig. 4.12) is named *Mac-1* because it was originally discovered in macrophages. The outside-in activation of this integrin stimulates a neutrophil to release primary and secondary granule contents into the infected stroma. The granules contain

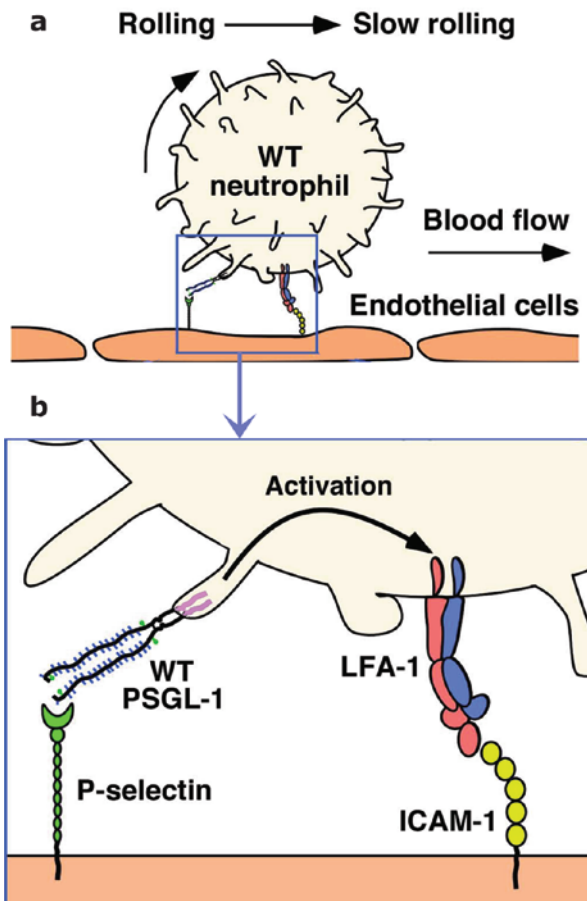


Fig. 13.7 Neutrophil activation. (a) *Neutrophil in the blood.* Neutrophils express P-selectin glycoprotein ligand-1 (PSGL-1; blue) and integrin $\alpha_L\beta_2$ (lymphocyte function-associated antigen-1, LFA-1; pink and blue). Endothelial cells constitutively express E- and P-selectins (dark green) and intercellular adhesion molecule-1, ICAM-1 (light green) when activated by IL-1, IL-8 and other cytokines. (b) *Neutrophil arrest and activation.* The PSGL-1/P-selectin interaction slows neutrophil rolling and activates its LFA-1 ($\alpha_L\beta_2$ integrin) to extend to an intermediate affinity conformation (i.e., inside-out integrin activation discussed in Chap. 4). If ICAM-1 is present, the LFA-1 extends fully and its ICAM-1 interaction arrests neutrophil at the endothelial cell surface (Figure from left side of Fig. 6 in Miner JJ, Xia, L, Yago T, et al. (2008 Sept 1) “Separable requirements for cytoplasmic domain of PSGL-1 in leukocyte rolling and signaling under flow.” *Blood*, 112(5):2035–45). This figure was modified by Dr. Wirsig-Weichmann

defensins, a group of small, cysteine-rich cationic proteins that destabilize bacterial cell membranes, peroxidase and lactoferrin (See Sect. 12.1.4), and proteins that activate macrophages and tissue-hydrolyzing lysosomal enzymes (Sect. 13.3.1).

During passage through the capillary wall, the neutrophil tertiary granules release neutral gelatinase (MMP-9) with which the neutrophils cut through type IV collagen in the

endothelial basement membrane. The neutrophils also secrete neutral collagenase (MMP8) and metalloelastase (MMP12) with which they cut through the collagen and elastin of capillary walls (Sect. 11.1.1) and the infected stroma. These matrilysins are all activated by plasmin, which is already present to prevent the clotting of plasma exuding from capillaries with the neutrophils. A tetracycline antibiotic derivative (doxycycline sold as *Periostat*) inhibits neutrophil collagenase activity at a tenth of the amount required for bacterial growth inhibition. Unfortunately, *Periostat*'s effect is limited because most collagen fiber loss is caused by acid-activated cathepsin K from macrophages (Sect. 13.3.1). *Periostat* is used in conjunction with therapy for periodontitis to facilitate reattachment and healing by limiting neutrophil collagenase action in the absence of cathepsin K.

13.2.5

Gingivitis is Reversible; Antiinflammatory Cytokines Mediate Repair

If oral hygiene is restored, the PAMPs mediating proinflammatory stimuli decrease. The surrounding, healthy keratinocytes produce a natural inhibitor of IL-1, IL-1 receptor antagonist (IL-1ra). IL-1ra competes with IL-1 for binding to the IL-1 receptor, closing capillary endothelial spaces. More importantly, the infiltrated leukocytes age and change to secreting antiinflammatory cytokines (lipoxins, resolvins, and protectins; Sect. 13.5.4) that promote healing and tissue repair. Nearby healthy cells respond to the antiinflammatory cytokines by releasing *fibroblast growth factor* (FGF) proteins. These proteins act on receptors that activate capillary growth (angiogenesis) and fibroblast secretion of hyaluronan into the spaces created by lost gingival collagen fibers (Sect. 6.3.1.). Collagenase action is inhibited by an anti-inflammatory cytokine-mediated increase in TIMPs (Sect. 8.1.3). Fresh collagen fibers are laid down, and a healthy gingival architecture is restored.

Interestingly, the secretion of FGF family members that mediate tissue repair resembles IL-1 secretion, that is, not involving the Golgi. The FGF family proteins also bind to their receptors by β -barrel structures that interact with a glycan (in this case known to be heparin) during activation. Hyaluronan and heparin are glycosaminoglycans (Sect. 6.3.1).

The successor microbiota is a major source of pathogen-associated molecular patterns (PAMPs), conserved prokaryotic motifs that are recognized by pattern recognition receptors (PRRs) in mammalian cells. These ligand-bound PRRs release interleukin-1 from the junctional epithelium and underlying tissues at the base of a sulcus. All cells have a receptor that is activated by binding to IL-1. The capillary endothelium receptor is especially sensitive. IL-1 receptors activate NF κ B to express a group of proteins which, in endothelial cells: (a) open intercellular junctions allowing plasma proteins to exude into the stroma; (b) secrete plasminogen activator to prevent the plasma from clotting; (c) induce ICAM-1 to arrest neutrophils and activate their extrusion from the capillaries; and (d) induce secretion of other proinflammatory cytokines. ICAM-1 binds to LFA-1, a β_2 integrin on the neutrophil surface where it induces a respiratory burst that

stimulates neutrophil migration into the stroma. A second integrin, Mac-1, is activated when the neutrophils contact collagen fibers in the stroma. Mac-1 activation facilitates the release of defensins and lysosomal enzymes that destroy bacteria. If oral hygiene is restored, the PAMPs are removed and the capillaries stop exuding neutrophils. The remaining neutrophils age and start releasing anti-inflammatory cytokines that stimulate the surrounding healthy cells into releasing fibroblast growth factor (FGF). Eventually, fresh collagen fibers are laid down, and a healthy gingival architecture is restored.

13.3.1

Long-term Effects of Persistent PAMP Stimulation

If oral hygiene remains poor, PAMPs keep stimulating IL-1 secretion and neutrophils continue to infiltrate the region. Another neutrophil product, *protease-3*, induces endothelial cells to secrete *monocyte chemotactic protein-1* (MCP-1), a chemokine resembling IL-8. It binds to a receptor on passer-by monocytes and induces an adhesion molecule similar to ICAM. Monocytes are arrested by double binding similar to neutrophils. MCP-3 functions like MCP-1, but it is secreted instead of MCP-1 by fibroblasts exposed to IL-1. MCP3 remains intact and functional in gingivitis because its cleavage requires fibroblast gelatinase, not leukocyte gelatinase (see Sect. 8.3.4); fibroblast gelatinase is not secreted.

The monocytes differentiate into macrophages and osteoclasts. The former enhance leukocyte (i.e., macrophage and neutrophil) destruction of the stroma by adding to neutral collagenase (MMP8) activity. MMP8 cleaves collagen fibers into one-quarter and three-quarter length tropocollagen fragments (Sect. 8.3.4). The leukocytes endocytose many partially digested collagen fragments into sealed compartments called phagosomes, which then fuse with lysosomal vesicles as in osteoclasts (Sect. 10.1.2). Cathepsin K is likely responsible for most of the gingival collagen lost in periodontitis, explaining why Periostat is only effective with other therapy (Sect. 13.2.4). Within phagolysosomes, lysosomal peroxidases and oxidases make *reactive oxygen species* (ROS) such as *hydrogen peroxide* that kills viable bacteria but also damages the surrounding gingival and periodontal tissues. Gingival and periodontal membrane cells restrict ROS production and repair the damaged cells (Sect. 16.3.2), but the ROS production from persistent leukocyte activation is so excessive that host cell and tissue damage cannot be prevented (Sect. 16.3.2).

Activated leukocytes also express IL-6, which increases the proliferation of antibody-producing lymphocytes (B-lymphocytes) locally and the replenishment of bone marrow neutrophils globally (Fig. 13.8). Another function of IL-6 is to act with IL-1 to increase the hepatic expression and plasma concentration of most blood clotting proteins (Sect. 11.1.1). This stimulation of blood clotting protein synthesis may *partially* explain the association of periodontitis with an increased likelihood of vascular clotting events, notably heart attacks and strokes (Sect. 13.1.2).

Activated leukocytes induce the synthesis and secretion from the liver of an unrelated set of proteins called acute-phase proteins. One such protein, *C-reactive protein* (CRP) was originally identified as binding to the phosphocholine attachment site of capsular polysaccharide (C-polysaccharide) from *Streptococcus pneumoniae*. CRP in blood has a half life of less than a day, compared with 4 days for fibrinogen. A continuously elevated

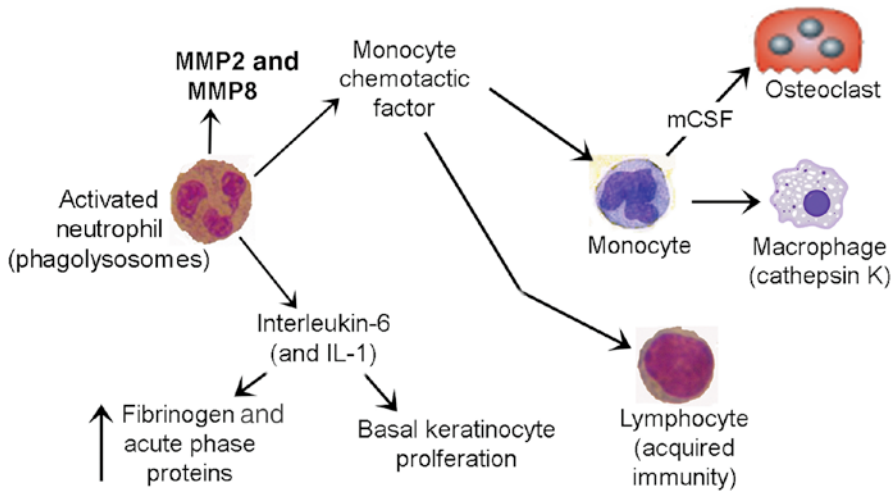


Fig. 13.8 Neutrophil activation products. An activated neutrophil secretes monocyte chemotactic factor (MCP) and an interleukin, IL-6. *Top right:* MCP attracts monocytes that differentiate into macrophages and osteoclasts. *Lower right:* MCP independently attracts thymic (T-) lymphocytes which are responsible for acquired, cell-mediated immunity. *Lower left:* IL-6 has systemic effects on blood plasma proteins and local effects on keratinocytes (Figure made up from Wikipedia public domain images except for osteoclasts and reorganized into an original figure <http://en.wikipedia.org/wiki/File:Neutrophil.jpg>; <http://en.wikipedia.org/wiki/File:Lymphocyte2.jpg>; <http://en.wikipedia.org/wiki/File:PBMonozyt.jpg>; <http://en.wikipedia.org/wiki/File:Macrophage.png>; Osteoclasts figure is from Fig. 4, p. 645 in Teitelbaum SL, Ross FP (2003 Aug) "Genetic regulation of osteoclast development and function." *Nature Reviews—Genetics* 8:638 – 649). This figure was modified by Dr. Wirsig-Weichmann

CRP content indicates a persistent proinflammatory stimulus in the body. CRP binds to host or bacterial phosphocholine, and the complex activates a group of plasma proteins called *complement* (Sect. 3.3.2). The complement system resembles the blood clotting system, except that proteolytic cleavage of its components results in peptide fragments that to attract and enhance the phagocytosis (*opsonization*) of CRP- or antibody-bound material by macrophages (like IL-1 or IL-8). CRP is part of the innate immune response, and the antibody response is part of the acquired immune response (Sect. 12.1.4). Many bacteria of the successor microbiota induce an antibody response that is increased in periodontitis compared with gingivitis or healthy subjects.

If oral hygiene remains poor, the neutrophils make a protease (protease-3) that releases monocyte chemotactic protein-1 (MCP-1) from endothelial cells. This protein behaves like IL-1 or IL-8, except that it acts on monocytes instead of neutrophils. In the tissues, the monocytes develop into macrophages and monocytes. Together with neutrophils, the macrophages engulf (phagocytose or opsonize) infected tissues into vesicles called phagosomes, which fuse with lysosomes to form phagolysosomes whose lysosomal component secretes acid and acid proteases as in osteoclasts. The phagolysosomes also

secrete defensins which disrupt the inner membrane of bacteria, and peroxidases and oxidases that make reactive oxygen species (ROS). The osteoclasts cause alveolar bone loss. Thus, periodontal pocket development and periodontitis are mostly due to the release of phagolysosome contents that remove gingival and periodontal membrane collagen fibers, demineralize alveolar bone, and reduce the viability of the junctional epithelium and surrounding cells. In addition, IL-6 secreted by neutrophils increases the plasma content of clotting proteins and C-reactive protein (CRP). CRP binds to phosphocholine on the surface of bacterial cells or of phagolysosome-damaged host cells and activates complement, a group of plasma proteins that undergo partial proteolysis into fragments. Some complement fragments attract and activate neutrophils like IL-8; others enhance the phagocytosis of CRP- or antibody-bound material by macrophages.

13.4.1 Apoptosis in Chronic Periodontitis

Mammalian cell death is classified as *autophagy*, *apoptosis*, or *necrosis*. Autophagy occurs when cells are starved of one or more essential metabolites and have to obtain them by internal digestion of their organelles and macromolecules. The cell shrinks until its contents are so depleted that it undergoes apoptosis, self-induced cell death due to internally activated *cathepsin proteases* (Table 8.1). In apoptosis, these cathepsin proteases are activated from within or externally and the cytosol is completely digested, followed by the cell membrane. Apoptosis is a biologically controlled procedure for getting rid of unwanted, damaged or infected cells which disappear without trace. Apoptosis occurs during organism development and is also activated by infection and various physicochemical agents.

By contrast, in necrosis, severe physical, chemical, or bacterial damage causes a cell membrane to burst, releasing apoptotic mediators (Sect. 13.4.3) and proinflammatory cytokines into the stroma (Sect. 13.2.2). The cytosolic enzymes continue to make lactic acid in the absence of mitochondrial function, making the necrotic environment strongly acidic and activating lysosomal enzymes (Sect. 13.2.1) to digest the released cytosolic contents. Necrosis is discussed in relation to aggressive periodontitis (Chap. 14).

In gingivitis, *lysine decarboxylase-induced autophagy* may impair the junctional epithelial attachment barrier to bacterial products (PAMPs) as gingivitis develops *de novo* or following therapy (Sect. 13.1.2). The dentally attached cells become autophagic and stop dividing if their lysine content becomes insufficient to support protein synthesis. A loss of basal laminar attachment to the tooth increases the permeability of junctional epithelium to PAMPs, and this activates PRRs (TLRs and NLRs) to release IL-1 (Sect. 13.2.1). Both TLRs and NLRs activate *caspase 1* (ICE, Sect. 13.2.2) by creating a cytosolic *inflammasome*, similar to the creation of a cytosolic *apoptosome* which activates different caspases to mediate apoptosis (Sect. 13.4.3).

In chronic periodontitis, apoptosis is caused by biofilm products. Products of asaccharolytic metabolism by the successor microbiota, most notably *butyrate* (Sect. 13.2), permeate the cell membrane of junctional epithelial cells at the base of the sulcus and induce apoptosis by specific intracellular interactions. Other PAMPs bind to NLRs in the cytosol and may mediate either apoptosis or inflammation (Sect. 13.2.1). In addition, leukocytes encountering teeth adherent microbial biofilm at the base of a sulcus undergo phagolysosome degranulation. The phagolysosome contents kill the bacteria (Sect. 13.3.1), but also junctional epithelial cells in the immediate vicinity. Apoptosis or mild necrosis of junctional epithelium at the base of the gingival sulcus is therefore reported in chronic periodontitis, but not in gingivitis. Necrosis of the gingiva, acute necrotizing gingivitis (ANUG), is discussed under aggressive periodontitis (Sect. 14.1.1).

13.4.2 Intracellular Induction of Apoptosis

Apoptosis is controlled by the Bcl-2 family of proteins whose nomenclature was derived from the first discovered protein of the family. This protein was encoded as the second gene within a segment of DNA that translocates from chromosome 11 to chromosome 14 in certain B cell lymphomas, *B cell lymphoma protein-2* (Bcl-2). The Bcl-2 proteins possess a tertiary structure that is common to pore-forming bacterial toxins, a hydrophobic helix surrounded by amphipathic helices (Fig. 13.9a). Each Bcl-2 family member shares at least one of four domains that bind with other family members. These *Bcl-2 Homology domains* are named BH1 through BH4 (Figs. 13.9a, b). Bcl-2 proteins that *effect* apoptosis such as BAX and BAK possess domains BH1 through BH3 (*BH4-missing effector proteins*). Proteins that *initiate* or *activate* the effector proteins possess a BH3 domain only (*BH3 domain-only proteins*), and those that prevent apoptosis, *antiapoptotic* like the founder member, possess all four domains.

Apoptosis is caused by the release or activation of one or more BH3 domain-only proteins (Fig. 13.9c, step 1). These *initiating* BH3-only proteins bind to a cytosolic heterodimer containing a Bcl-2 protein (step 2) and displace a previously bound BH3-only protein, Bid, BIM or PUMA (step 3). In the case of Bid, dissociation from a partner such as Bcl-2 may be mediated by proteolysis of the Bid N-terminal 60 amino acid residues, creating truncated Bid (tBid), which activates an *effector* protein. The BH3-only proteins are therefore *activators of apoptosis* that displace effector proteins (BAK or BAX) from a non-Bcl-2 partner on the cytosolic surface of mitochondria and endoplasmic reticulum (step 4). For example, BAK is attached to an outer mitochondrial membrane channel protein in a healthy cell. An activating BH3-only protein displaces BAK, which then self-aggregates into homodimers that burrow a hole in the mitochondrial or endoplasmic reticular membrane, releasing the contents (step 5).

Induction by interactions between Bcl-2 family proteins may be induced by cytokine-receptor binding (*extrinsic*) or by intracellular changes (*intrinsic*).

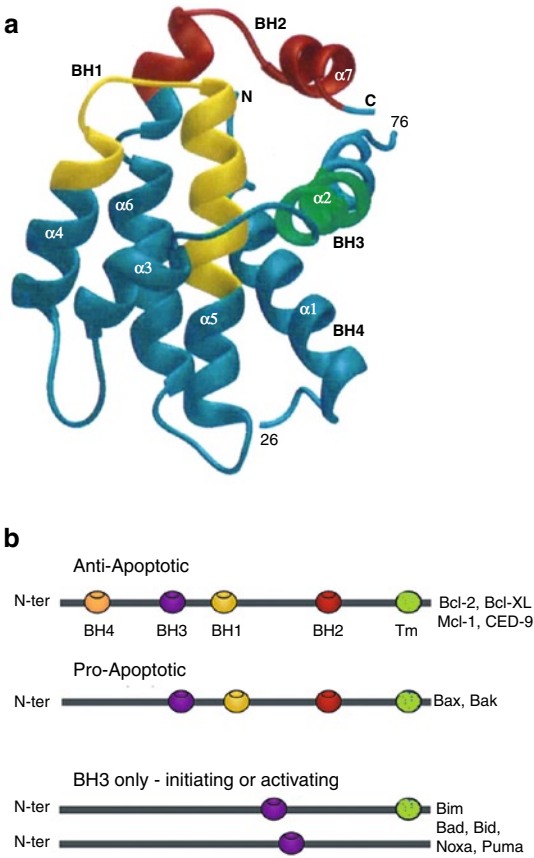


Fig. 13.9 Domain structure and interactions among Bcl-2 family proteins. **(a)** *Structure of a Bcl-2 family member; Bcl-XL*. There are six α -helices (blue, numbered 1 through 6) whose side chains mediate partner binding and/or insertion into a membrane. The Bcl-2 homology domains (BH domains) are labeled BH1, BH2, and BH3 regions (yellow, red, and green colored ribbon, respectively, corresponding to amino acid regions 86–100, 129–148 and 180–195). The BH4 region is coincident with the α 1 helix (amino acids 4–24) **(b)** *Bcl-2 family member domain organization*. Bcl2 family members have four, three or one of the Bcl-2 Homology (BH) domains. A common sequence motif identifies each domain and a unique sequence motif each domain number. Bcl-2 family members with only three BH domains are BH4 domain-lacking proteins (the apoptosis effector proteins). Bcl-2 family member with only one domain are BH3 domain only proteins and divided into initiating and activating as indicated below (**a**: Figure 1c from Muchmore SW, Sattlert M, Liang H, et al. 1996, May 23, “X-ray and NMR structure of human Bcl-X_L, an inhibitor of programmed cell death.” Nature 381:335–341; **b**: Wikipedia public domain image (http://en.wikipedia.org/wiki/Image:Bcl-2_Family.jpg)

13.4.3 Mechanisms of Apoptosis

The extrinsic pathway mechanism is mediated by cytokine ligands: the *TNF-related apoptosis-inducing ligand* (TRAIL), the *Fas ligand* (FasL), and other homologous cytokines. All these ligands bind to receptors homologous to the $\text{TNF-}\alpha$ receptor. Like $\text{TNF-}\alpha$, FasL and TRAIL are present in the cytosol of most cells and are released by the ligand-bound receptor as unstable homotrimers (Sect. 3.2.2). For apoptosis to occur, the bound ligand must pool the membrane domain of the receptor into clusters of lipids (lipid rafts) on the cell surface. The clustering allows the C-terminal domains of the receptors to associate and form *TNF receptor-associated death domains* (TRADD) or *FAS receptor-associated death domains* (FADD).

The TRADD or FADD domains bind to adapter proteins in the cytosol and together they form of a *death inducing signaling complex* (DISC). This complex recruits caspase-8, one of a family of *calcium ion-activated serine proteases* (caspases) which cleave polypeptides on the C-terminal side of their aspartate residues. DISC activates caspase-8 to activate a Bcl-2 activator, BID (Fig. 13.10a). The resultant tBid fragment activates effector Bcl-2 proteins on the endoplasmic reticular membrane and mitochondrial outer membrane.

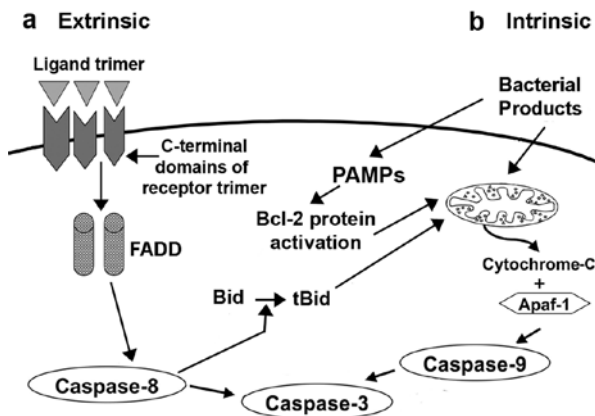


Fig. 13.10 Caspase activation by extrinsic and intrinsic apoptotic pathways. **(a) Extrinsic path.** This path is mediated by members of the tumor necrosis factor (TNF) superfamily (see text). **(b) Intrinsic pathway.** This path may be mediated by various physiological or pathological changes, but only the modes of induction of this path in periodontitis are discussed (see text). Details of cytochrome c release and caspase activation are shown in Fig. 13.11 (This figure is modified from Fig. 1 in Schimmer AD (2004 Oct 15) "Inhibitor of Apoptosis Proteins: Translating Basic Knowledge into Clinical Practice." *Cancer Research*, 64:7183–7190)

Calcium ions are released into the cytosol from the endoplasmic reticulum and cytochrome c and other proteins from the mitochondrial intermembrane space. The calcium ions activate caspase-9, enhancing the activation of caspase-3, which is activated by caspase-8 in addition to BID.

In chronic periodontitis, the intrinsic apoptotic pathway (Fig. 13.10b) may involve short-chain fatty acids and other bacterial products penetrating into the cytosol of junctional epithelial cells at the base of a sulcus. Some NLR proteins may activate Bcl-2 effector proteins, or exposure to the contents of phagolysosomes that have degranulated (Sect. 13.3.1) may cause membrane leakage. In either case, soluble proteins from the mitochondrial intermembrane space and calcium ions from the endoplasmic reticulum and extracellular fluid pass into the cytosol. Cytochrome c is released into the cytosol where it reacts with *apoptotic protease activating factor-1* (apaf-1) and calcium ions to activate caspase-9 and caspase 3 (Fig. 13.11a). Apaf-1 possesses an N-terminal *caspase recruitment domain* (CARD) that interacts with a matching CARD on caspase 9 (Fig. 13.11a). The exposed CARD domains in the cytochrome c/Apaf-1 complex aggregate to form an apoptosome (Fig. 13.11b) whose central hub contains attached caspase-9. The bound caspase-9 auto-activates and frees itself from the apoptosome by cleaving off its pro- and CARD domains.

In addition to cytochrome c, a *second mitochondria-derived activator of caspases* (SMAC) is also released from the mitochondrial intermembrane space. SMAC binds to an *inhibitor of apoptosis protein* (IAP) to prevent spontaneous caspase activation in a healthy cell. The IAP that binds to caspase-9 is called X-IAP. When SMAC binds to X-IAP, X-IAP is released from caspase-9. Autocleavage of the caspase-9 prodomain then exposes the catalytic site. Caspase-9 then activates caspase-3 which immediately cuts out the X-IAP binding domain from procaspase-9, preventing X-IAP inhibition and accelerating activation of both caspase-3 and caspase-9 (Fig. 13.11a), which digest all of the cell's proteins. The apoptotic cells also possess a *caspase-activated DNase* (CAD) that fragments chromosomal DNA. Other proteins from the mitochondrial intermembrane space can activate caspases by related mechanisms. The combination of mechanisms, or the overwhelming activation of any one of them, irreversibly propels a cell into apoptosis.

Mammalian cell death is classified as autophagy, apoptosis, or necrosis. In gingivitis, autophagy may impair the barrier to bacterial products (PAMPs). In chronic periodontitis, apoptosis occurs in junctional epithelium. Bacterially produced short chain fatty acids permeate the cell membrane and activate NOD-like proteins that mediate apoptosis, or phagolysosomes degranulate on contacting the biofilm. Both mechanisms activate Bcl-2 family proteins to punch holes in endoplasmic reticular and mitochondrial membranes, releasing calcium ions into the cytosol along with cytochrome c and other proteins from the mitochondrial intermembrane space. Cytochrome c reacts with apoptotic protease activating factor-1 in the cytosol to form an apoptosome complex that binds to and activates caspases, calcium ion-activated serine proteases that cleave on the C-terminal side of aspartic acid residues in polypeptides. In healthy cells, inhibitors of apoptosis proteins (IAPs) block the catalytic site of spontaneously activated caspases.

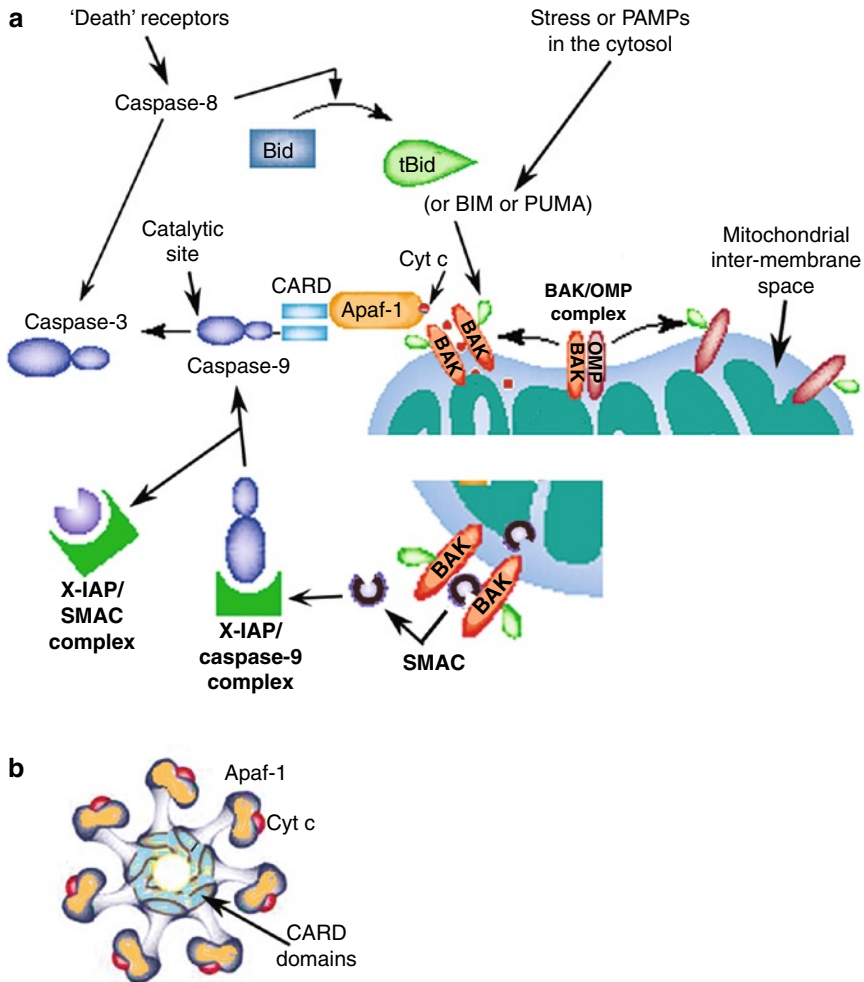


Fig 13.11 Mitochondrial intermembrane space molecules in apoptosis **(a)** *Activation creates a pore in the mitochondrial membrane:* BAK or BAX create a pore (hydrophilic hole) in the membrane allowing cytochrome c (red dots) or SMAC (purple crescent) to enter the cytosol from the mitochondrial intermembrane space. BAK and BAX are also present with a partner on the endoplasmic reticulum and their activation similarly releases Ca^{2+} ions into the cytosol (not illustrated). Cytochrome c interacts with Apaf-1 (orange oval) whose CARD domains (blue rectangles) bind to caspase-9. Caspases also bind to cytosolic inhibitor of activation proteins called IAPs (see text). X-IAP is the specific IAP that binds to caspase-9. **(b)** *The apoptosome:* The central CARD domains of aggregated Apaf-1 proteins form a pinwheel-like apoptosome that activates caspase-9 (see text) **(a):** Figure is modified from Fig. 2 in Joza N, Kroemer G and Penninger JM (2002) "Genetic analysis of the mammalian cell death machinery." Trends in Genetics 18 (3):142–149; **b:** Figure is taken from Fig. 1 in Danial NN, Korsmeyer SJ (2004 Jan 23) "Cell death: critical control points." Cell 116(2):205–219). This figure was modified by Dr. Wirsig-Weichmann

13.5.1

Eicosanoids and Periodontal Repair

Correct tooth brushing, mouth cleansing, and flossing is the primary defense against chronic periodontitis, but nearly always insufficient. Regular visits to a dentist are extremely important to remove calculus from the tooth surface. Once periodontitis is present, therapy begins with a procedure, called scaling and root planning in which the gingival and root surfaces of the teeth are scraped clean using ultrasonic and hand instruments. The teeth are then polished and the patient is instructed in oral hygiene to slow the redevelopment of plaque and calculus. If the disease is moderate or severe, one quadrant is treated per week and the scaling and root planning is accompanied by minor surgery to reconstitute the gingival architecture. This therapy facilitates the disappearance (*resolution*) of the inflammation and the restoration of a healthy sulcus. Removing the successor microbiota immediately stops any apoptosis or necrosis of DAT cells and reduces the bacterial inflammatory stimulus (Sect. 13.4.1).

The process of resolution (described in Sect. 13.2.5) is directed by a subgroup of *eicosanoids*, a family of signaling molecules found in virtually all tissues and organs. They are synthesized from *polyunsaturated fatty acids*, fatty acids with multiple double bonds (Fig. 13.12) that are essential components of cell membranes. These fatty acids must be obtained from the diet because they cannot be synthesized *de novo* in the mammalian body. Their function is to prevent membrane hardening, especially in cold water fish for the same reason that less hydroxyproline allows physiological dissociation of collagen fibers (Chap. 4). In polyunsaturated fatty acids, the carboxyl group (COO⁻) is always numbered carbon atom 1 (C1), and the double bond nearest to the terminal (omega, ω) carbon atom is 3 or 6 carbon atoms distant (omega-3 or omega-6; Fig. 13.12).

13.5.2

Eicosanoid Structure

Eicosanoids are made up of six families: prostaglandins, thromboxanes, leukotrienes, and lipoxins from arachidonic acid (omega-6); resolvins from eicosapentaenoate or docosahexaenoate (omega-3); and protectins from docosahexaenoate (omega-6). Prostaglandins were originally identified as hormone-like compounds secreted by the prostate gland and thromboxanes as blood coagulation factors secreted by activated platelets. Their inflammation promoting and resolving activity were subsequently discovered in related compounds. The arachidonate-derived eicosanoids are made by reacting arachidonate with *cyclooxygenases*, which are present in most cells, or *lipxygenases*, which are present mostly in leukocytes (granulocytes and macrophages). There are two *cyclooxygenase* (COX) *isoenzymes*, one constitutive (COX-1) and the other induced by cytokines (COX-2), and together they give rise to the prostaglandins and thromboxanes. There are also three lipxygenase isoenzymes, each with slightly different specificity and expressed mainly by granulocytes and macrophages.

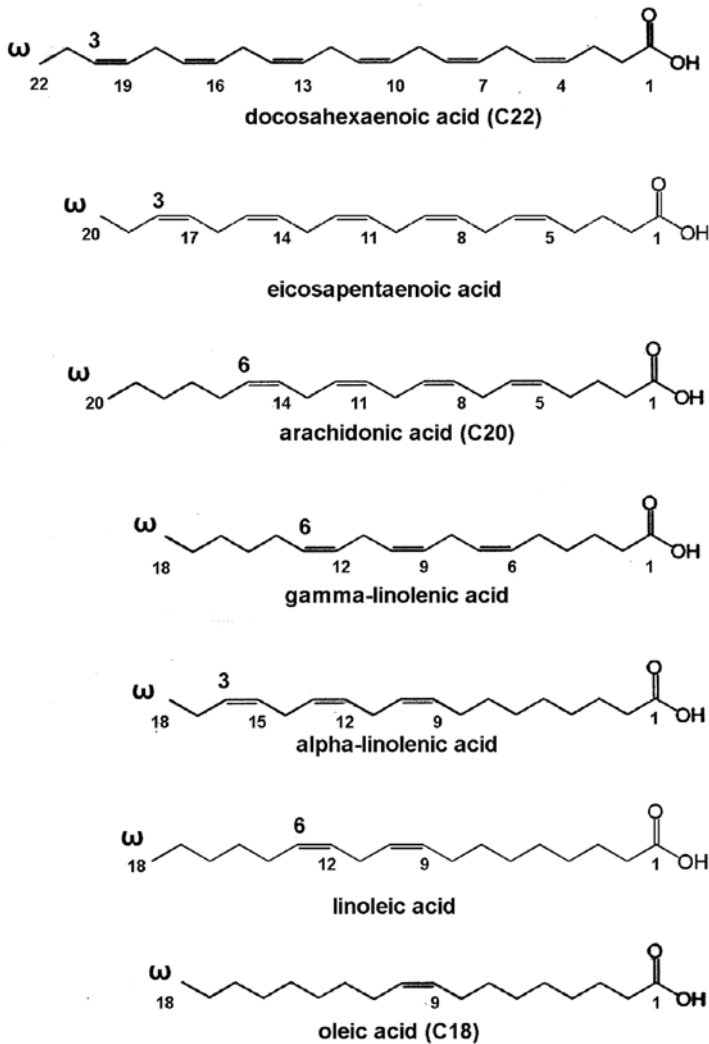


Fig. 13.12 Polyunsaturated fatty acids required for eicosanoid synthesis. Oleic acid is the only fatty acid synthesized by mammals *de novo*. Linoleic (ω-3) and α-linolenic acid (ω-6) cannot be synthesized, because mammals have a desaturase enzyme that only makes ω-9 or greater fatty acids. Ingested ω-3 fatty acids are metabolized to other ω-3 fatty acids with ω-9 double bonds. The same applies to ω-6 fatty acids. The major dietary sources of polyunsaturated fatty acids are fish and plants oils

The two COX enzymes have ~60% identical amino acid sequences. They both exhibit two enzymatic activities: cyclooxygenase activity, which converts arachidonic acid to prostaglandin G_2 ; and peroxidase activity which reduces a hydroperoxide on prostaglandin G_2 to the corresponding alcohol, prostaglandin H_2 (Fig. 13.13a). The COX enzymes possess *dioxygenase* and *peroxidase* centers that are interdependent. The peroxidase center

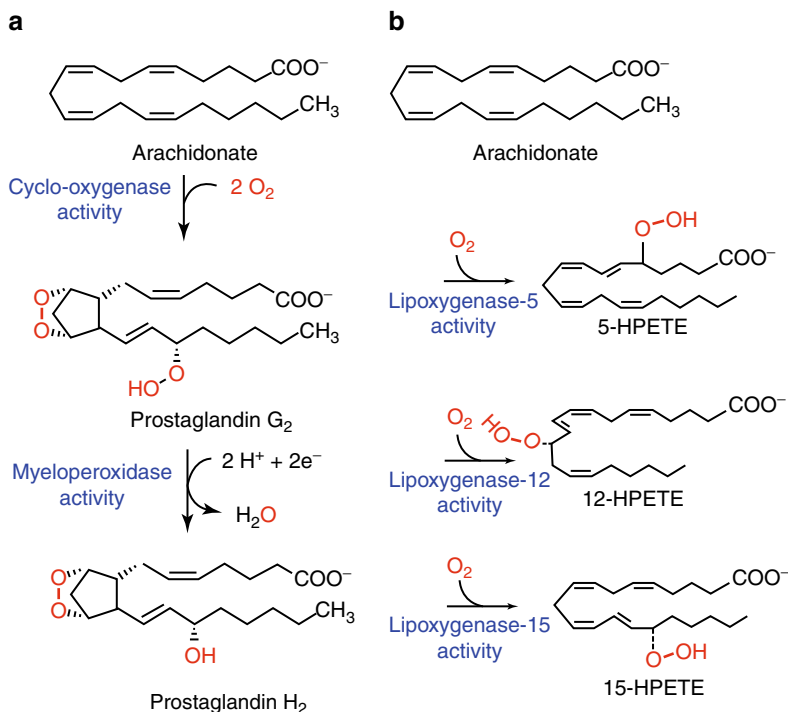


Fig. 13.13 Cyclooxygenase and lipoxygenase action on arachidonic acid. **(a)** *Cyclooxygenase mechanism.* There are two forms of cyclooxygenase, constitutive and inducible. Both possess a cyclooxygenase reaction center, which adds two oxygen molecules (in red) per molecule of arachidonate. One oxygen molecule forms a cyclic ring involving three carbon atoms and the other forms a peroxide at carbon atom 15 (C15). The product (prostaglandin G_2) is immediately attacked by a separate, interactive myeloperoxidase reaction center on the cyclooxygenase polypeptide. The myeloperoxidase reduces the C15 peroxide to a hydroxy group. **(b)** *Lipoxygenase action.* Leukocytes may oxidize arachidonate at C5, or C12 or C15 with one of three lipoxygenase isoenzymes to give arachidonate 5 hydroperoxide (hydroperoxyeicosatetraenoate, 5-HPETE, etc.). The peroxy group is converted to the corresponding hydroxyeicosatetraenoate (5-HETE etc.) by a peroxidase enzyme and is then oxidized further by an appropriate lipoxygenase and/or hydrated by a hydrolase to give the intermediates and end-products indicated in Fig. 13.10 **(a):** Figure from <http://www.jungfreudlich.de/wp-content/uploads/2007/08/formation-of-prostaglandin-h2.jpg>; **b:** Figure from <http://www.benbest.com/health/Lipoxygenase.jpg>

possesses a Fe^{2+} /heme cofactor related to myoglobin that attaches two molecules of molecular oxygen to activate the dioxygenase-catalyzed oxidation and reduction of bound arachidonic acid. One molecule of oxygen (O_2) forms a ring between carbons 9 and 11 (C9 and C11) of arachidonate and the other attaches as a peroxy group to carbon 15 (C15). Because the two reactions are inseparable, COX1 and COX 2 are also known as the *prostaglandin H synthetases* (PGHSs). Prostaglandin H_2 is the precursor of all other prostaglandins or thromboxanes (Fig. 13.13a), which are made by further cyclooxygenase action determined by tissue type.

Lipoxygenases are Fe^{2+} -containing dioxygenases that catalyze the hydroperoxidation of arachidonate and other unsaturated fatty acids to form leukotrienes, lipoxins, resolvins, or protectins. They are attached to the cytosolic side of membranes by calcium ions. There are various lipoxygenase isoenzymes, of which three make leukotrienes or lipoxins by adding a peroxy group to a specific carbon atom of a double bond in arachidonate, C5, C12 or C15 (Fig. 13.13b). The peroxy group is enzymatically reduced to a hydroxyl group ($-\text{OH}$) by a separate peroxidase enzyme. Lipoxygenases are unusual in requiring two identical substrate molecules for activity. The active site of lipoxygenases contains a single iron atom bound (coordinated) to three histidine residues. The iron attaches a molecule of oxygen to each of two polyunsaturated fatty acid substrates and then transfers two electrons to form two peroxy groups, one on each substrate. Lipoxygenases are regenerated by electrons from glutathione by the pathway described in Chap. 6 (Fig. 6.8).

13.5.3

Functions of the Proinflammatory Eicosanoids

Cytokines or hormones release arachidonate or other fatty acid substrate from the cytosolic side of phospholipids by activating phospholipase A_2 . For example, leukotriene B_4 (LTB_4) is synthesized in neutrophils in which arachidonate is released and lipoxygenases induced by IL-1 or IL-8. Like IL-8, LTB_4 induces the adhesion of leukocytes to capillary endothelium, activates them, and promotes their movement into the infected stroma (Sect. 13.2.3). LTB_4 is also a potent chemoattractant for neutrophils in which it induces the formation of reactive oxygen species and the secretion of lysosomal (acid-activated) enzymes. Macrophages secrete prostaglandin E_2 , the osteoclast activating factor responsible for alveolar bone loss in periodontitis. The varied actions of eicosanoids on target cells are mediated by receptors that functionally resemble cytokine receptors.

Prostaglandins are secreted by a *prostaglandin transporter* (PGT) protein. This single-polypeptide contains 12 transmembrane regions within the plasma membrane of most cells and tissues. PGT proteins transport small negatively charged solutes through a membrane in response to chemiosmotic ion gradients. These transporters belong to the *organic-anion-transporting polypeptide* (OATP) class of proteins, a subgroup of the *major (transport) facilitator superfamily* (MFS). These transporters are unrelated to the ABC or drug export proteins that are involved in transporting large proteins such as Aa leukotoxin (Sect. 14.2.2).

13.5.4

Lipoxygenase-Mediated Resolution of Inflammation

Resolution is accompanied by a switch away from the cyclooxygenases (prostaglandins, thromboxanes) to lipoxygenases (lipoxins, resolvins, and protectins). Each has a different substrate: arachidonate for lipoxins, eicosapentaenoate for resolvins, and docosahexaenoate for protectins (Fig. 13.12). Each class therefore requires a different lipoxygenase isoenzyme. The first peroxy group is always added to the 15C atom and then peroxy groups are

In chronic periodontitis, a proinflammatory response is maintained by the continued binding of PAMPs in the junctional epithelium and gingival stroma. If PAMPs are not entirely removed, a fresh neutrophil infiltrate replaces any aged neutrophils that are synthesizing lipoxins, resolvins, and protectins. Thus, a balance between resolution and enhancement of inflammation develops, depending on how much of the microbiota remains at any time. Resolvins and protectins have three major roles. (a) Inhibit nuclear factor- κ B activation (Sect.10.2.2); (b) Recruit monocytes that differentiate into modified macrophages that exit into the lymphatic system after phagocytosis of microorganisms, apoptotic neutrophils and other cell debris without producing proinflammatory mediators; and (c) Stimulate the expression of acquired and innate immunity molecules (Sect. 12.1.4).

13.5.5 Antiinflammatory Drugs

The inhibitors of COX enzymes are called *nonsteroidal antiinflammatory drugs* (NSAIDs) that are prescribed to relieve pain and fever. They stop prostaglandin and thromboxane production. Acetylsalicylic acid (aspirin) was used for this for many years and it was eventually discovered to acetylate a serine residue involved in the dioxygenase action of cyclooxygenases. A second class of NSAIDs, typified by ibuprofen (commonly called Advil or Motrin), inhibits catalysis by attaching irreversibly to cyclooxygenases. Aspirin and ibuprofen inhibit all prostaglandin and thromboxane synthesis.

Both classes of these drugs inhibit important functions of constitutive prostaglandins and thromboxanes: an adequate secretion of stomach mucus and blood clotting. Mucous protects the stomach epithelium from acid ulceration and bleeding. Ibuprofen causes stomach bleeding by stopping mucus secretion and aspirin inhibits thromboxane A_2 synthesis, which down-regulates the platelet activation required for blood clotting (Sect. 11.2.1). Unfortunately, a combination of aspirin and ibuprofen reduces the usefulness of aspirin in preventing clotting without decreasing the incidence of stomach bleeding.

NSAIDs that preferentially inhibit COX-2 more than COX-1 (Vioxx and Celebrex) target the pain associated with inflammation such as rheumatoid or osteoarthritis with fewer stomach problems because prostaglandin I_2 is synthesized by COX-1 in the stomach. In the heart, continuous muscular movements cause capillary wear. Cytokines induce COX-2 to make prostaglandin I_2 which dilates the capillaries and prevents excessive blood clotting by thromboxane A_4 , a COX-1 enzyme product. A COX-2 inhibitor stops prostaglandin I_2 synthesis. The heart capillaries do not dilate and there is a greater risk of coronary artery obstruction (heart attacks).

Some studies suggest that COX inhibitors might control chronic periodontitis after therapy. The inhibition of COX would reduce neutrophil activation and emigration to the affected site.

By removing the successor microbiota, therapy and oral hygiene facilitate the disappearance (resolution) of inflammation, the regeneration of the apical portion of a damaged periodontium and the restoration of a healthy junctional epithelial attachment. Resolution is directed by eicosanoids, derived from omega-3 or omega-6 polyunsaturated fatty acids which are dietary essential components of cell membranes. Proinflammatory cytokines release free polyunsaturated fatty acids that activate inducible and noninducible classes of cyclooxygenase (COX-1 and COX 2) to synthesize proinflammatory prostaglandins and thromboxanes. In addition, lipoxygenases or peroxidases add peroxy groups to arachidonate, giving rise to proinflammatory leukotrienes. As leukocytes age, lipoxygenases change their specificity to making lipoxins instead of leukotrienes from arachidonate. Omega-3 fatty acids larger than arachidonate are also released as additional lipoxygenase substrates for the synthesis of additional antiinflammatory eicosanoids (resolvins and protectins). Lipoxins resolvins and protectins stop neutrophil infiltration by inhibiting nuclear factor- κ B activation and activate the recruitment and differentiation of monocytes into phagocytes (modified macrophages) that: (1) remove cell debris without stimulating proinflammatory mediators; (2) increase exit of these phagocytes from the inflamed site to the lymphatics; and (3) stimulate the expression of innate and acquired immunity. Nonsteroidal antiinflammatory drugs (NSAIDs) reduce prostaglandin and thromboxane production by irreversibly binding to or acetylating a COX enzyme. Inhibition of COX-1 stops stomach mucus secretion and promotes erosions that bleed because of the simultaneous inhibition of thromboxane A_4 . Other NSAIDs inhibit COX-2 enzyme activity but also prostaglandin I_2 , which dilates physiologically damaged capillaries in the heart. Capillary obstruction occurs because thromboxane A_4 , from COX-1 remains active. The antiinflammatory effects of NSAIDs might control chronic periodontitis.

This chapter discusses generalized and localized aggressive periodontitis. The generalized form is mediated by genetic defects or perhaps by altered cytokine or hormonal responses to stress making the gingiva exceptionally sensitive to products from the successor microbiota (Sect. 1). The localized form is primarily caused by infection of the gingival sulci by *Aggregatibacter actinomycetemcomitans* (Sect. 2).

14.1.1. Generalized Aggressive Periodontitis

Aggressive periodontitis occurs in less than 0.1% of the US population. Some forms of aggressive periodontitis are clinically different from chronic periodontitis, whereas others differ mainly in the rapid rate at which the attachment loss progresses. Like chronic periodontitis, aggressive periodontitis may be localized or generalized. The microbial etiology of the localized disease is well understood, whereas that of generalized aggressive periodontitis is not. The latter may accompany certain collagen- or fiber-maturation enzyme-related mutations (Table 7.1), or acquired or genetic defects of neutrophil function such as genetic mutations of the integrin α_L or α_M subunits (Sects. 13.2.3 and 13.2.4), or alterations in leukocyte responsiveness associated with systemic cytokine stimulation in stress, diabetes, or heart disease.

One form of generalized aggressive periodontitis is *Acute Necrotizing Ulcerative Gingivitis (ANUG)*, clearly a misnomer. The junctional epithelial attachment with its underlying cells and collagen fibers is rapidly destroyed along with coronal alveolar bone (see definition of coronal in Sect. 3.1.5). The amount of attachment lost in 10 days may take many years to occur in chronic periodontitis.

A key feature of ANUG is *necrosis* of sulcular and junctional epithelial cells and of fibroblasts and osteoblasts in the underlying stroma. Alveolar bone may be exposed in the oral cavity and the resulting pain usually leads a patient to seek help. In necrosis, the

plasma membrane ruptures (Sect. 3.4.1), causing a release of apoptotic and inflammatory agents into the extracellular medium. TRAIL and FAS mediate extrinsic apoptosis of adjacent cells (Sect. 13.4.2) and TNF α and IL-1 mediate a powerful release of pro-inflammatory cytokines from more distant cells. The exact cause of ANUG is unknown, but it usually appears in individuals who have been severely stressed. Hormonal responses to severe stress alter the junctional epithelial cells response to TLR or NOD receptor activation by PAMPs from the successor microbiota (Sect. 13.2.1).

During ANUG, acid and lysosomal enzymes are released into the extracellular environment and acid activated. Cathepsin L hydrolyzes uncalcified collagen fibers, and cathepsin K hydrolyzes calcified collagen fibers due to osteoclast activation. Cathepsin L is homologous to cathepsin K but has a different specificity. In necrotic tissues, cathepsin L *cleaves the nonhelical telopeptides regions of types I and II collagen*. These collagen peptides are absorbed and passed to the bloodstream where they provide a unique blood plasma marker for necrotic bone destruction (Sect. 4.2.2). Cathepsin L is also involved in the macrophage digestion (processing) of foreign material (antigens) during the acquisition of immunity.

Aggressive periodontitis occurs in less than 0.1% of the US population. ANUG is a form of aggressive periodontitis in which stress hormones may overactivate TLRs to PAMPs around and within cells at the base of gingival sulci. The junctional epithelium fragments and its cells release their contents (*necrosis*). The necrotic cell contents induce a massive release of pro-inflammatory cytokines that cause apoptosis of adjacent cells and a heavy infiltrate of leukocytes from more distant cells. Neutrophils and monocytes invade and destroy the surrounding periodontal attachment and alveolar bone.

14.2.1.

Localized Aggressive Periodontitis

Localized aggressive periodontitis (LAP) occurs as deep pockets around the first erupted teeth of children and young adults, i.e. their permanent central incisors and first molars. The LAP pockets extend well into the periodontium and contain large amounts of a small gram-negative rod, *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* (*Aa*). This bacterium expresses two soluble protein toxins: a *Leukotoxin (Ltx)* and a *Cytolethal distending toxin (Cdt)*. Most *Aa* isolates express relatively low levels of Ltx and have been designated minimally leukotoxic, but several express elevated levels. *Aa* is present in plaque (biofilm) from about 75% of East Asian populations (China and Thailand), about 50% in Africans and Hispanics, and about 25% in Caucasians. The greater prevalence of LAP in these populations is believed due to a cultural environment that promotes increased *Aa* exposure, although genetic (racial) differences cannot be discounted. About half of US individuals with LAP have an otherwise symptomless defect of neutrophil function.

Aa leukotoxin lyses leukocytes (neutrophils, monocytes, and macrophages) on contact, whereas *Cdt* kills lymphocytes whose surface antigen receptor has bound to an antigen, or macrophages whose Mac1 integrin has bound to one of its many ligands (Sect. 13.2.4). In LAP, antibodies that bind to both these toxins appear in the blood and they may facilitate

the rapid removal of these toxins after infection, preventing attachment loss from spreading beyond the first infected sites (the first six permanent teeth to appear in the oral cavity). These antibodies inhibit leukotoxin but not cytolethal distending toxin activity *in vitro*.

14.2.2.

Aa Leukotoxin Composition and Properties

Aa leukotoxin belongs to the *RTX (Repeats in toxin) family* of proteins, important virulence (damaging or poisonous) agents secreted by Gram-negative bacteria. The repeat sequence in these proteins is a domain of 6–40 repeats of a 9 amino acid sequence. In Aa leukotoxin, 14 such repeats form 7 calcium ion binding domains (Fig. 14.1a). Removing calcium ions from the extracellular medium, or expressing a mutant in which the repeat regions are absent results in an inactive leukotoxin. Two classes of RTX toxins are cytolytic (i.e. they lyse the cells of an infected host): hemolysins and leukotoxins. Hemolysins and leukotoxins cause erythrocytes and leukocytes to lyse (necrosis), hemolysins primarily on erythrocytes and leukotoxins primarily on leukocytes. In both cases the mechanism is similar. The toxin causes the membrane of the affected cell to burst and cytosolic contents are released into the extracellular fluid, stimulating necrosis of the target cells and inflammation of surrounding cells. Aa leukotoxin mainly targets human neutrophils.

The repeats which characterize Aa leukotoxin and other RTX family members are present in lipases and metallopeptidases related to serravalysin in various Gram-negative bacteria (Sect. 8.1.1), and in anthrax toxin lethal factor (Fig. 8.1) and diphtheria toxin in Gram-positive bacteria. The Gram negative RTX toxins are all secreted using a type I secretion path (Chapter 1, Sect. 1.4.2). This path involves two other proteins (protein B and protein D) and an activating enzyme (protein C), all encoded with the leukotoxin (protein A) in an operon, the *ltx* operon. The activating enzyme is an acyl transferase enzyme, which transfers a fatty acid to an internal lysine residue. Transfer of this fatty acid is from an acylated Acyl-Carrier Protein (acylACP) in the bacterial cytosol to the leukotoxin (Fig. 14b). Mutations or inhibitors of the transferase cause the protein to be secreted without leukotoxic activity.

Leukotoxin secretion is mediated by proteins B and D assisted by protein TdeA, which is encoded elsewhere in the genome (Fig. 14.1b). TdeA and TolC, its homologue in *E. coli* strains possessing an LTX hemolysin, are members of a family of proteins that enhance drug resistance by transporting antibiotics out of bacterial cells. *The name of the Aa protein hints at the drug-export function, Toxin and drug export protein A (TdeA)*. Protein B is an *ATP-Binding Cassette* transporter (ABC-transporter), one of a large and ancient family of transmembrane proteins that bind to ATP on the cytosolic face of a membrane and use the energy of ATP hydrolysis to transport a substrate to the extracellular face. Protein D is a multimeric membrane fusion protein whose channel spans the entire inner membrane and periplasm (intermembrane space). The protein D multimers therefore attach protein B on the cytosolic side to a TdeA-formed channel in the outer membrane (Fig. 14.1a).

As noted above, proteins B and D are encoded downstream of the acylase (protein C) and the leukotoxin (protein A) genes and their expression is attenuated in comparison with upstream-encoded proteins. The amounts of proteins B and D are reduced compared with

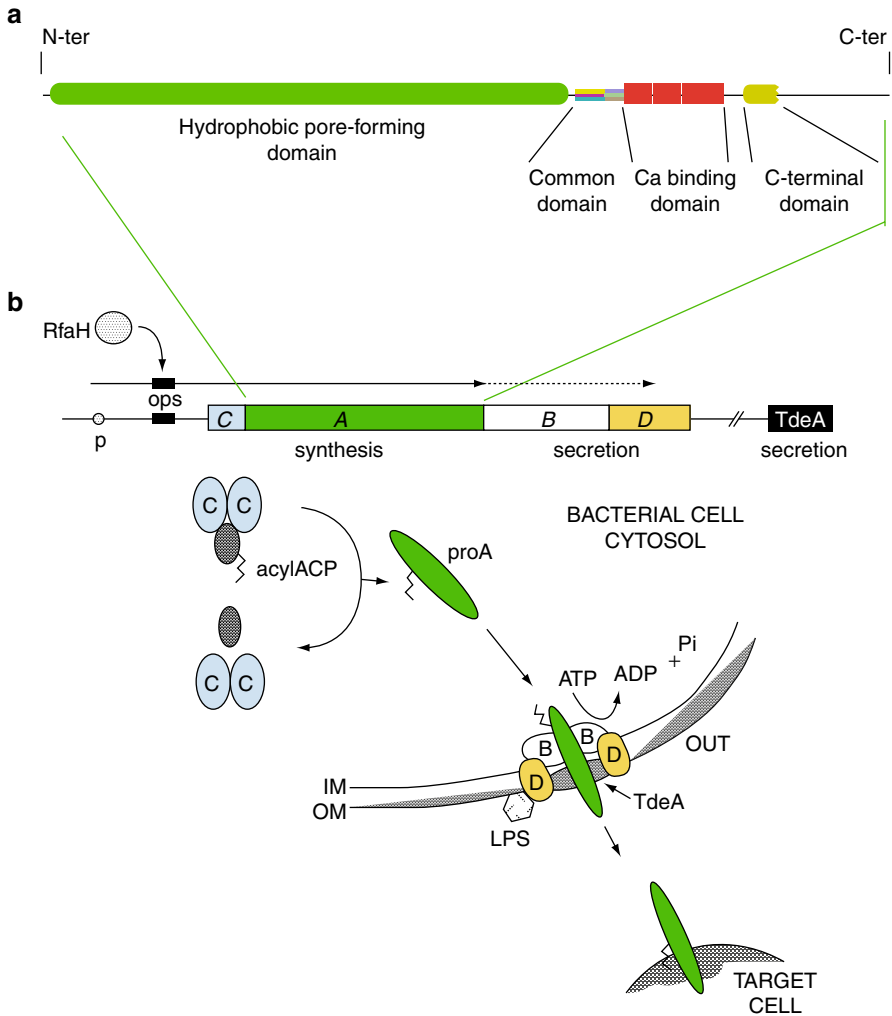


Fig. 14.1 Structure and synthesis of Aa leukotoxin. **(a)** *Polypeptide domain structure.* The polypeptide is 1,050 amino acid residues in length. The pore-forming region is the large N-terminal domain (dark green; residues 5–654) plus the short C-terminal domain (light green; residues 757–774). The large N-terminal domain is followed by a region that is common to all RTX proteins. It consists of two domains, one multicolored (yellow, red, and blue; residues 660–695) and one colored blue only (residues 696–720) and a tandem repeat sequence (red; residues 721–846) from which the protein family takes its name (see text). Each repeat contains the Ca^{2+} ion binding consensus sequence: GGXGXDX Φ X where Φ is a hydrophobic residue and X is any amino acid (see text). **(b)** *Synthesis and secretion.* The *lktCABD* operon is responsible for the synthesis, maturation, and secretion of Aa leukotoxin (protein A; green). The promoter sequence (p) is shown along with an operon polarity suppressor sequence (*ops*) and a termination suppressor protein (RfaH) that binds to the *ops* nucleotide sequence and facilitates expression of the downstream proteins B (white) and D (yellow). Proteins B and D are expressed at lower levels due to transcription termination within the operon and are required to secrete the

the amounts of proteins A and C. Less proteins B and D are required because they induce secretion of the acylated leukotoxin by forming a complex spanning the inner and outer cell membranes. This complex recruits TdeA by conformational changes and then transports the acylated leukotoxin, unfolded and rapidly, to the outer the cell surface where it folds to a native conformation, is released and binds calcium ions (Fig. 14.1b).

After secretion, the acylated, calcium-adherent leukotoxin attaches to the β_2 integrin of LFA-1 that extends out from the surface of activated neutrophils. The leukotoxin binding occurs near the N-terminus at the propeller region (Chapter 4, Sect. 4.4.1), possibly to N-linked oligosaccharides. The bound leukotoxin undergoes a conformational change that exposes its hydrophobic surface and causes its insertion into the neutrophil membrane. At low doses, the leukotoxin increases calcium levels in the cytosol, activating caspases and apoptosis by the intrinsic pathway (Fig. 13.10). At high concentrations, pores form in the cell membrane and the cell contents empty into the stroma (necrosis).

14.2.3.

Mutations Enhance Aa Ltx and LAP Severity

In the operon encoding the leukotoxin (*ltx* operon), proteins B and D are encoded downstream of the acylase (protein C) and the leukotoxin (protein A) genes. In long bacterial RNA transcripts, such as the hemolysin (*hly*) operon in *E. coli*, the expression of proteins B and D is normally attenuated when compared with that of the first encoded proteins C and A. A similar attenuation occurs in the Aa *ltx* operon, except in LAP strains, which possess. In Aa strains isolated from subjects with localized juvenile periodontitis, there is often a 530 base pair deletion between the *ltx* promoter sequence and the first nucleotide of the RNA transcript. This deletion causes the promoter sequence (TATAAT) to lie much closer to the translation start site (ATG encoding the first amino acid of protein C). The 530 base pair deletion includes the C-terminal part of an open reading frame, *orfA*, a fifth protein expressed from the transcript along with the 4 proteins of the *ltx* operon (Fig. 14.1b, up-arrow on the far left side). The



Fig. 14.1 (continued) leukotoxin. Prior to secretion, a lysine residue near the C-terminal end of the pore-forming domain (possibly lys 555, which corresponds to lys 565 in the homologous *E. coli* hemolysin) is acylated with an unknown fatty acid. A second acylation site (lys 691 in the *E. coli* hemolysin) is absent from Aa leukotoxin. Acylation precedes secretion into the extracellular medium, after which Ca^{2+} ion binding to the leukotoxin repeat sequences activates the toxin. TdeA is an outer membrane protein homologous to TolC and encoded by a gene outside the *ltxCABD* operon (see text) (a: From <http://pfam.sanger.ac.uk/protein?acc=P16462>; b: Adapted from Fig. 1 in Stanley P, Koronakis V, and Hughes C (1998) "Acylation of *Escherichia coli* hemolysin: A unique protein lipidation mechanism underlying toxin function." *Microbiology and Molecular Biology Reviews* 62(2):309–333. Modified for Aa as described by Crosby JA and Kachlany SC (2007) "TdeA, a TolC-like protein required for toxin and drug export in *Aggregatibacter (Actinobacillus) actinomycetemcomitans*." *Gene* 388:83–92. With permission)

deleted portion of the OrfA protein encodes a basic amino acid sequence that likely binds to DNA. In the many Aa strains without this deletion, the OrfA protein may control *ltx* operon protein expression by an unknown mechanism. Deletion of this protein moves the *ltx* operon promoter region closer to the translation start site and enhances leukotoxin expression.

Unlike chronic periodontitis, Aa does not form biofilms (plaques) around the affected teeth. Aa strains have a “rough” or “smooth” appearance when grown on blood agar plates. When grown on liquid medium, the “rough” strains adhere tightly to the glass surface of the cultivation tube whereas the “smooth” strains adhere only slightly. The lack of adherence is due to mutations in *tad* genes that encode proteins homologous to the type II and type IV secretion proteins required for pilus formation (Sect. 1.4.2). These nonadherent *tad* mutants secrete copious amounts of leukotoxin in vitro, whereas the wild-type strains (with “rough” morphology and tight adherence) secrete very little leukotoxin (which remains attached to the outer cell membrane). *Tad* gene mutations and the 530 base pair deletion of OrfA protein in the *ltx* operon may have a common cause. Both types of mutations enhance leukotoxin expression and/or secretion and associate with severe LAP.

14.2.4.

Cytolethal Distending Toxin (Cdt)

Cdt is related to a eukaryotic cytosolic enzyme, phosphatidyl inositol-3,4,5, triphosphate 5-phosphatase which removes the 5-phosphate group from phosphatidyl inositol-3,4,5, triphosphate. This activity is part of an intracellular signaling cascade induced by a ligand binding to a nearby receptor. Phosphatidyl inositol-3,4,5-triphosphate 5-phosphatase possesses an *Src Homology 2* (SH2) domain in addition to its *Inositol Phosphatase* activity (SHIP).

The SH2 domain is a conserved sequence in a viral tyrosine kinase responsible for transforming fibroblasts into neoplastic sarcoma cells (the viral *src* gene, *v-src*; see sect. 10.2.1. for the normal cell counterpart, *c-src*). SH1 is a tyrosine kinase domain; SH2 is the domain that binds to phosphotyrosine residues; and SH3 is a *v-src* domain that binds to proline-rich sequences in proteins. SH2 and SH3 domains are found in many proteins involved in signal transduction.

SHIP is primarily expressed in bone marrow hemopoietic and lymphoid precursor cells. It is especially important in lymphocytes whose cell surface receptors have bound to an antigen, and in macrophages that have bound to lipopolysaccharide by TLR-4 (Sect. 13.2.1). The phosphatidylinositol-3 kinase (PI3 kinase) pathway generates PI-3,4,5-triphosphate. As it accumulates, PI-3,4,5-triphosphate activates SHIP in a feedback loop to reduce PI-3,4,5-triphosphate concentration on the inner membrane. In nonleukocytes, antigen and TLR receptors are absent, and PTEN (*Phosphatase of the tensin* family of phospholipid phosphatases) is activated instead of SHIP. The name PTEN is derived by its first being found deleted from chromosome 10 in certain cancer cells. PTEN reduces membrane PI-3,4,5-triphosphate by removing the 3-phosphate instead of the 5-phosphate (Fig. 14.2). PI-3,4,5-triphosphate is a second messenger molecule that stimulates cell survival, adhesion, migration, metabolic activity, proliferation, differentiation, and end cell activation by various pathways (not shown in Fig. 14.2) and its amount is continuously adjusted in response to the environment.

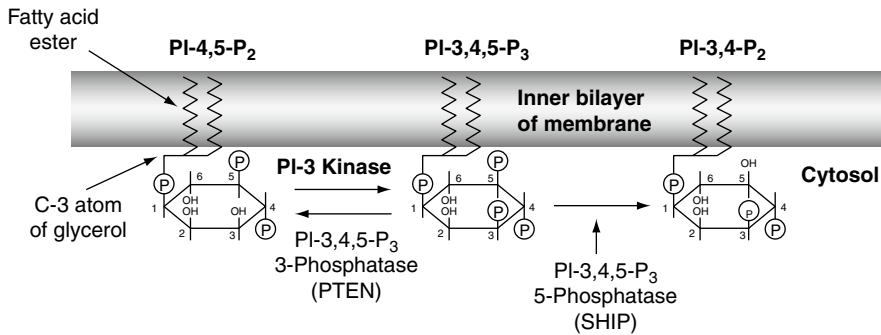


Fig. 14.2 Mode of action of cytolethal distending toxin. Phosphatidylinositol is attached to the inner layer of a cell membrane by its fatty acids. Inositol is a six-membered carbon ring with an OH group on each carbon atom. The atoms of the inositol ring are numbered counter-clockwise from the C1 atom, which is attached to a membrane diacylglycerol phosphate by the C3 atom of the latter's glycerol moiety. The C2 and C1 atoms are attached to fatty acids. Inositol phosphate is phosphorylated by membrane kinases at the -4 and -5 positions and then the -3 position to make phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P₃), which activates growth and various biological processes. In most cells, production of PI-3,4,5-P₃ is kept low by the enzyme PTEN (see text), but in macrophages SHIP is activated to remove the 5-phosphate (see text). Cytolethal distending toxin (Cdt) is a homologue of SHIP and therefore competes for PI-3,4,5-P₃ only in activated leukocytes; other cells are less sensitive (see text) (Modified from Fig. 1 (p. 1171) in Sly LM, Rauh MJ, Kalesnikoff J, et al. (2003) "SHIP, SHIP2, and PTEN activities are regulated in vivo by modulation of their protein levels: SHIP is up-regulated in macrophages and mast cells by lipopolysaccharide." *Experimental Hematology* 31:1170–1181. With permission) Edited by Foxit Ready Copyright (c) by foxit software company, 2005–2008. for Evaluation only.

Cdt is composed of three proteins, all encoded by the *cdt* operon. Two of these proteins bind to human cell membranes where they facilitate the translocation of the third subunit, a SHIP-like phosphatase PI-3,4,5-trisphosphate 5-phosphatase. This bacterial phosphatase passes through the membrane and removes the 5-phosphate from PI-3,4,5-trisphosphate, causing stimulated lymphocytes and macrophages to undergo apoptosis instead of growth. In nonleukocytic cells such as epidermal cells or fibroblasts, PI-3,4,5-phosphatase is exposed to PTEN, which is 10 times more active on PI-3,4,5-trisphosphate than Cdt. The SHIP-like bacterial enzyme therefore cannot compete with PTEN. The sensitivity of nonleukocytic cells to Cdt is much less than that of stimulated lymphocytes and macrophages.

Localized aggressive periodontitis is self-limiting but it predisposes to generalized chronic periodontitis if untreated. It appears around the gingival sulci of the first erupting permanent teeth due to infection with *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* (*Aa*). This bacterium expresses two soluble protein toxins, a leukotoxin (Ltx) and a cytolethal distending toxin (Cdt). Both affect leukocytes, but induce systemic antibodies that may prevent the periodontitis from spreading to later

erupting teeth. Aa leukotoxin belongs to the RTX family of proteins, found in other Gram-negative bacteria. Ltx is encoded along with other proteins by the *ltx* operon: these are *OrfA*, a putative protein that controls operon expression; an acyl transferase that adds a fatty acyl group essential for leukotoxin activity; and two proteins that facilitate secretion. Aa leukotoxin is activated by its repeat regions binding Ca^{2+} ions in the extracellular medium. It binds to the LFA-1 integrin of neutrophils and forms pores in the neutrophil cell membrane. High expression is associated with deletion mutations of *OrfA*, or point mutations of *tad* genes responsible for adherence and biofilm formation. LAP is associated with an absence of tooth adherent biofilms and high Ltx expression. Cytolethal toxin (Cdt) is encoded by Aa along with two other proteins in the *Cdt* operon. In humans, two of the encoded proteins bind to cell membranes and translocate the third, a phosphatase that inactivates a growth promoter, phosphatidyl inositol-3,4,5 triphosphate (PI-3,4,5-P_3) by removing its 5-phosphate group. Lymphocytes and macrophages are unable to grow and ultimately apoptose. Nonleukocytic cells possess a 3-phosphatase, which competes for this substrate better than Cdt, making nonleukocytes resistant.

This chapter describes dental caries (tooth decay) and its causes. Sucrose and other mono- and disaccharides are metabolized to acid (lactate) by bacteria that remain in “stagnation” areas of the teeth. Rats and hamsters fed a 50% sucrose diet developed a caries-sensitive, predominantly gram-positive microbiota that became caries resistant when the rodents were fed penicillin (Sect. 1). Further studies identified *Streptococcus mutans* (*S. mutans*) as the etiological agent. This organism synthesizes an insoluble polysaccharide capsule that is stable and retains lactate at the enamel surface (Sect. 2). The key enzyme, glucosyl transferase, is related to salivary amylase which adheres to oral bacteria and enhances bacterial acid production. The chapter concludes with a discussion of salivary and other factors responsible for the marked variation observed in individual caries experience (Sect. 3).

15.1.1.

Dental Caries: Definition and Measurement

Dental caries, cavities or tooth decay, provides dentists with an ongoing demand for preventive and reconstructive services. Caries is of Greek origin; it means destruction or decay. Dental caries refers to the dissolution of tooth enamel and dentin. It starts in the pits, fissures, and interdental regions of the teeth, “stagnation areas” from which bacteria are difficult to remove (Fig. 15.1). The extent of caries is measured as the number of teeth diagnosed as decayed, missing, or filled due to caries, the Decayed, Missing and Filled Teeth (DMFT) index.

15.1.2.

Sugar, Dental Caries, and the Dental Profession

Because teeth are preserved long after death, it was evident that European populations suffered little from caries before the late eighteenth century. The rise of caries coincided with increased sucrose consumption; perhaps, the revenge of black slaves on their masters. Before the civil war ended in 1863, slaves from Africa were forced to cut cane sugar in the

Fig. 15.1 Severe, untreated dental caries. Dental caries initially affects the occlusal pits and fissures of posterior teeth. In severe caries, the smooth surfaces, especially the interdental surfaces of all teeth may be involved



West Indies and far Southern US. From the late eighteenth century and throughout the nineteenth century, increasing amounts of the raw cane were exported to Atlantic-facing ports of England and Scotland. There, in sugar refineries, it was crushed, cleaned, and the fluid crystallized to give pure sucrose, *refined sugar*. During this period, new methods increased the yields of corn, wheat, and other grains and their starch was purified (refined) to add to various foods. *Pure starch and sucrose are called refined carbohydrate*. Populations in Europe, Canada, and the Northern US became addicted to refined carbohydrate because of the cold climate. The dramatic increase in caries throughout the nineteenth and early twentieth centuries led to the development of local and general anesthetics and of the dental profession to treat the associated infections, pain, and discomfort.

In 1945, the *Vipeholm study* was set up to determine whether increasing sucrose intake actually increased human caries experience. The marked variation in caries severity between individuals (Sect. 15.2.1) had led to doubts that increased sucrose consumption was really responsible. The study was conducted at the Vipeholm Hospital for individuals with mental handicaps outside the University City of Lund, Sweden. The study examined the effects of different diets on dental caries in the inmates and it ended in 1951. The results indicated that sticky sugar candies (toffees) between meals and popular with the inmates increased their DMFT by an average of one cavity per year. The use of mentally handicapped subjects was criticized in the Swedish press and all studies on mentally handicapped individuals were stopped in 1954.

Another series of studies was conducted on the inmates of *Hopewood House*, Bowral, New South Wales, Australia. This home for children of unmarried mothers opened in 1942 and closed in 1965. The children were made to eat a diet of locally grown raw vegetables with a minimal amount of protein in the form of milk and raw soybeans. The almost complete absence of dental caries compared with other Australian children was noted in studies conducted between 1955 and 1960. Unfortunately, as soon as they entered the general population as adults, all former inmates developed caries rapidly. Moreover, a harrowing description of what this diet was like, and of many other violations by the owners and employees of this orphanage, was given to the Australian Senate by Sandra Pendergast in 2003. In November 2009, the Australian government issued a formal apology to those who had grown up suffering abuses that were common to this and many other orphanages in Australia.

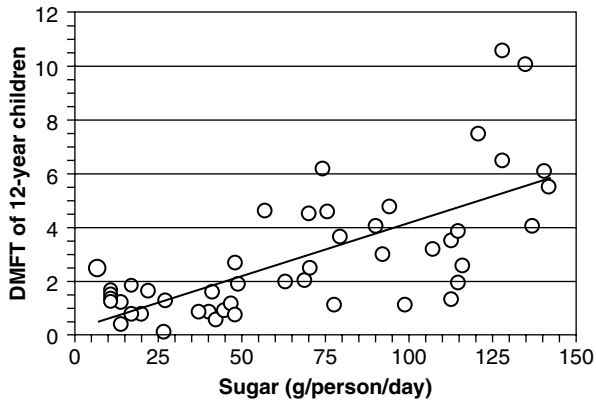


Fig. 15.2 Relationship between dietary sucrose intake and dental caries severity. The number of teeth that were decayed, missing, and filled due to caries (decayed, missing, and filled teeth [DMFT]) is graphed against mean sugar consumption in 12-year old children. Each point on the graph represents a different country. The findings were available from World Health Organization activities in oral epidemiology (The graph was assembled by the author from Table 3 in L. M. Sreebny (1982) “Sugar availability, sugar consumption and dental caries.” *Community Dentistry and Oral Epidemiology* 10:1–7)

Today, cane sugar is mostly grown and exported throughout the world from the eastern half of Africa south of the Equator, and from India and Indonesia. The most compelling evidence for sucrose causing caries was obtained by comparing the sucrose intake in 12-year old children from 47 countries (obtained from the World Health Organization’s Global Oral Epidemiology Bank during the 1970s) with the DMFT during this period (Fig. 15.2). The number of affected teeth increased by about one DMFT for every 25 g of sugar consumed daily. About 50% of the variance in DMFT between populations is due to the daily sucrose intake.

15.1.3.

Sucrose and the Appearance of Acid in Dental Biofilms

Bones and teeth dissolve in acid. The insoluble calcium monophosphate salt, from which hydroxyapatite is made, is converted to the more soluble calcium dihydrogen phosphate salt in an environment whose pH is less than 6.2 (Sect. 9.1.1). The severity of caries was related to the pH produced in dental biofilms (plaques) after ingesting sucrose and other sugars by Richard M Stephan. The pH response he identified is referred to as Stephan Curve. He found that the starting pH, the extent of its drop, and the time for recovery to the starting pH were all related to caries severity. The pH drop was later associated with lactic acid production due to bacterial carbohydrate fermentation (saccharolytic fermentation, Sect. 1.3.2). The subsequent rise in pH was due to the production of ammonia by bacterial

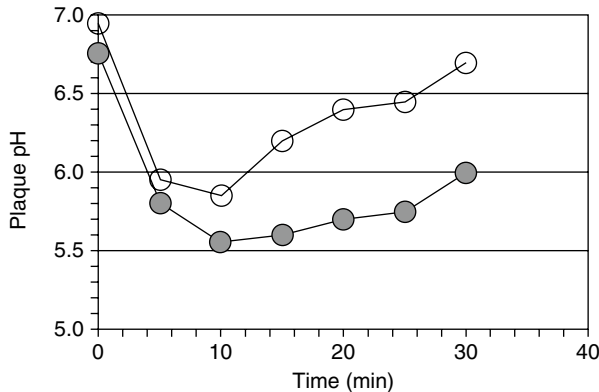


Fig. 15.3 Biofilm (plaque) pH in caries-resistant and caries-susceptible subjects. The pH decreased and returned to initial values more rapidly following a 10% sucrose rinse in caries-resistant subjects (○) than in caries-susceptible subjects (●). Each group had five subjects (Figure 2 in Mandel and Zengo (1973) “Genetic and chemical aspects in caries resistance.” Comparative immunology of the oral cavity, ed. H. Scherp and S. Mergenhagen, pp. 118 – 137, US Government Printing Office)

asaccharolytic fermentation after the sucrose was depleted (see Sect. 15.3.1). The curve was originally shown to occur after a 10% glucose mouth rinse, but a sucrose mouth rinse produces a similar result (Fig. 15.3).

15.1.4. Cavities in Animals and *Streptococcus mutans*

In rodents, caries induction was unpredictable until a high-carbohydrate low-fat diet (~60% sucrose plus soy and milk proteins plus vitamins and minerals) was developed. Germ-free rodents fed this diet did not develop caries and normal rodents (rats, hamsters, and mice) also did not develop caries when their diet was supplemented with penicillin (0.01–0.05% in the drinking water). Penicillin inhibits the growth of gram-positive bacteria by interfering with cell wall formation (Sect. 1.4.1). Young mice fed the diet without penicillin developed rampant caries within 35 days, but no caries in the presence of penicillin. If the penicillin was removed from the drinking water after one generation, the mice produced several generations of progeny that developed little caries despite the diet, suggesting that the absence of gram-positive bacteria resulted in a stable microbiota associated with caries resistance. These experiments are illustrated in Fig. 15.4.

In 1960, Robert Fitzgerald and Paul Keyes demonstrated the types of bacteria responsible for caries. They isolated streptococci from a carious lesion and made several of the isolates resistant to a non-penicillin antibiotic (streptomycin) which provided them with a means of isolating and identifying these bacteria subsequently. The streptomycin-resistant strains were used to infect young hamsters that were resistant to caries despite the diet. The infected hamsters developed caries and the streptomycin-resistant bacteria appeared in

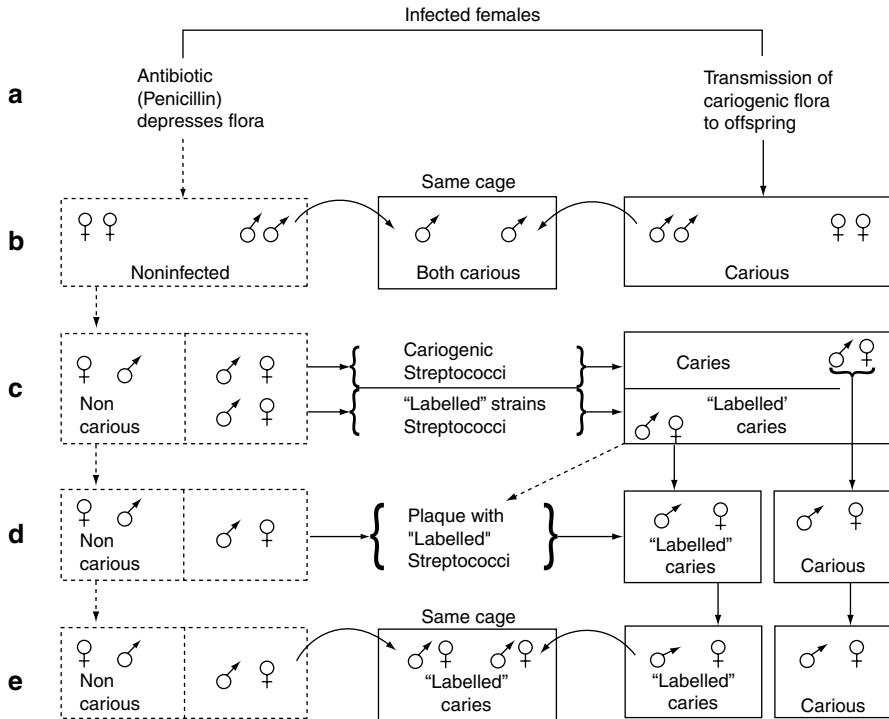


Fig. 15.4 Identification of a cariogenic microbiota – the Fitzgerald–Keyes experiments. (a) *Penicillin inhibits bacterial colonization*. When penicillin was added to the drinking water, gram-positive bacteria were depleted (depressed) and caries did not develop. (b) *Caries is transmissible*. After a generation, penicillin was no longer provided, but the offspring remained caries resistant to the sucrose diet for many generations (*left side* of figure). The caries-resistant rodents developed caries only if they were placed in the same cage as the animals that did develop cavities (not receiving penicillin). (c) *Adding streptococci to the caries-resistant microbiota causes caries*. Strains of what were later called mutants streptococci were isolated. Some were labeled by being made streptomycin resistant. Oral inoculation of the unlabeled or streptomycin-resistant strains into caries-resistant animals caused them to develop caries. (d) *Adding streptomycin-resistant "labeled" strains to biofilm (plaque) from caries-resistant males or females cause caries*. (e) *These streptococci cause caries even if obtained from feces*. The streptomycin-resistant streptococci in feces smeared on the body of a caries-resistant rodent caused the animal to develop caries (Figure 3 in Fitzgerald RJ and Keyes PH (1965 Oct) "Dental caries as a major disease problem." *Medical Annals of the District of Columbia* 34(10):463–467)

their oral cavity and feces. These bacteria remained absent from separately caged caries-resistant animals fed the same diet in the same room. Thus, the caries-susceptible microbiota is composed of streptococci and is transmitted by direct contact, not aerosols (Fig. 15.4). The streptococci were later identified as *Streptococcus mutans*. Although a caries-resistant microbiota is stable in the presence of a sucrose diet, the procedures required to develop and maintain it are impracticable for human populations. Removing sucrose from the diet or adding fluoride to the diet markedly decreased caries development in rodents possessing the cariogenic microbiota (flora).

Dental caries (cavities or tooth decay) led to the development of the dental profession by the late nineteenth century. It is caused by refined carbohydrates, pure sucrose, and starch in the diet. Sucrose between meals, especially sticky candies, increased cavities in most adults, and raising children on a diet without refined carbohydrate prevented caries development. A World Health Organization study showed that the average number of carious teeth in populations of 12-year old children from 47 countries increased by one for every 25 g of sugar consumed daily. Experiments in rodents confirmed that a high sucrose diet caused caries and that the responsible microbiota was sensitive to penicillin. Administering penicillin to the drinking water protected the rodents from developing caries despite the diet. Caries resistance was transmitted after removing penicillin by keeping the offspring isolated from caries-susceptible rodents despite the diet. The caries-resistant rodents developed caries if they came into direct contact with penicillin-sensitive streptococci subsequently identified as *Streptococcus mutans* from caries-susceptible rodents.

15.2.1.

How Sucrose Connects *S. mutans* to the Oral Microbiota and Dental Caries

By the late 1960s, the rodent studies had led to dental caries being generally accepted as caused by refined carbohydrate promoting a saccharolytic gram-positive microbiota that adhered to teeth surfaces. Mutans streptococci were considered key components of this microbiota. The name “mutans” is derived from the Latin word for change, “mutatio.” On agar, *Streptococcus mutans* grows as small round colonies in the presence of glucose, but as large, sticky, gelatinous colonies in the presence of sucrose. The gelatinous colonies are caused by a capsule around the bacteria: mutan, an $\alpha 1 \rightarrow 3$ glucose polymer (Fig. 15.5), and dextran, an $\alpha 1 \rightarrow 6$ glucose polymer. Mutan and dextran are glucans (made from glucose) like starch, glycogen, and cellulose. Functionally, the most important portion of the *S. mutans* capsule is its mutan. The aqueous solubility of a glucan depends on its major bonds; cellulose ($\beta 1 \rightarrow 4$) and mutan ($\alpha 1 \rightarrow 3$) are mostly insoluble, whereas dextran ($\alpha 1 \rightarrow 6$), glycogen, and starch ($\alpha 1 \rightarrow 4$) are mostly soluble.

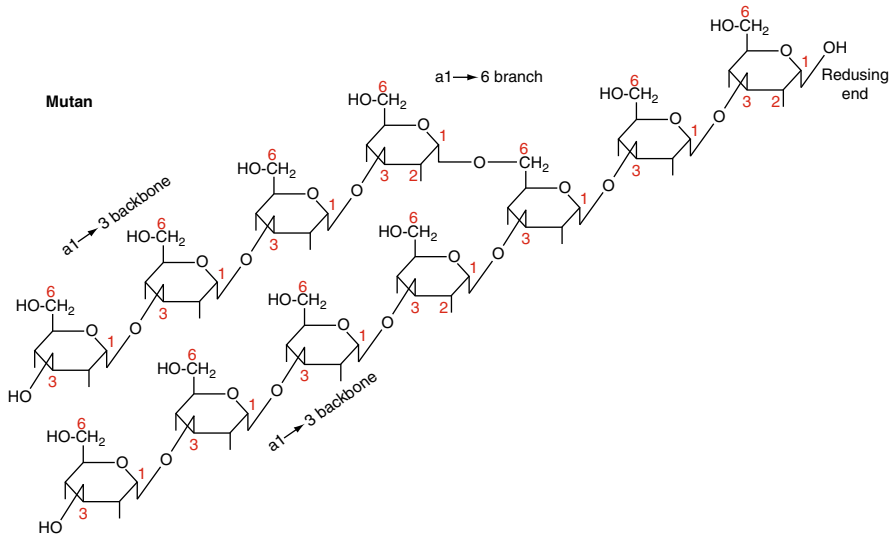


Fig. 15.5 Structure of mutan. Diagram shows the glucose pyranose ring with extensions indicating the OH group positions. The carbon atoms are numbered. The residues are linked together by $\alpha 1 \rightarrow 3$ bonds with $\alpha 1 \rightarrow 6$ branching (shown) and occasional $\alpha 1 \rightarrow 2$ branching (not shown). The reducing end is at the *top right*. Mutan is the product of GTF-I

In sucrose, the glucose and fructose moieties are connected by their respective anomeric carbon atoms (Fig. 15.6). The alpha anomer predominates in glucose, whereas the beta anomer predominates in fructose (indicated by the larger size of the predominant form in Fig. 15.6 c compared with b). The hydrolysis of sucrose releases two anomeric carbon atoms that provide the energy for bacterial capsule formation at the stagnation areas where caries develops. Glucan capsules (i.e. mutan and dextran) are made from the glucosyl moiety of sucrose. Some oral bacteria produce instead a capsule called levan from the fructose moiety of sucrose (Fig. 15.7). The enzymes that synthesize these polysaccharides, *glucosyl-* and *fructosyl-transferases*, are not exclusive to oral bacteria.

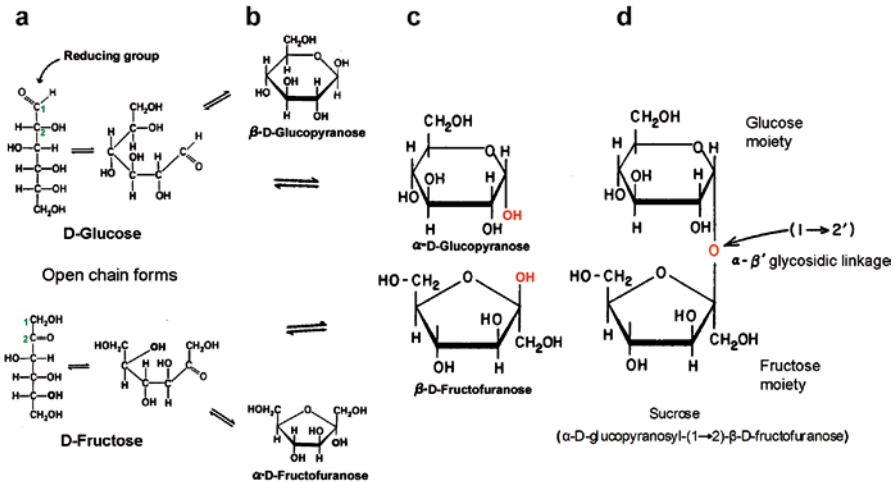


Fig. 15.6 Structure of sucrose. Sucrose is a disaccharide composed of glucose and fructose. **(a) Ring structures.** In glucose, the ring is formed from an aldehyde at carbon 1 of the molecule (*top*). The C1 carbon is the reducing end and it possesses the anomeric -OH group, α - pointing down or β - pointing up in the D series sugars (see legend to Fig. 6.7). In fructose, the ring is formed from a ketone at C2 of the molecule (*bottom*). The reducing carbon is C2 and the anomeric -OH group is attached to C2. **(b) Anomers.** The usual glucose anomer is the α -form and the usual fructose anomer is the β -form. These dominant anomers are drawn larger than the respective uncommon anomers. **(c) Anomeric forms incorporated into sucrose.** The usual anomeric forms of glucose and fructose create the sucrose glycoside (anomeric -OH groups in red). **(d) Structure of sucrose.** The two anomeric -OH groups form the glycoside bond of sucrose (O-glycoside atom colored red). The structure is drawn to make the fructose glycoside comparable with maltose (see Fig. 12.10). The sucrose diagram in Fig. 2.9 has the fructose rotated, causing its glycoside bond to appear α -anomeric, whereas it is in fact a β -anomer as in this diagram

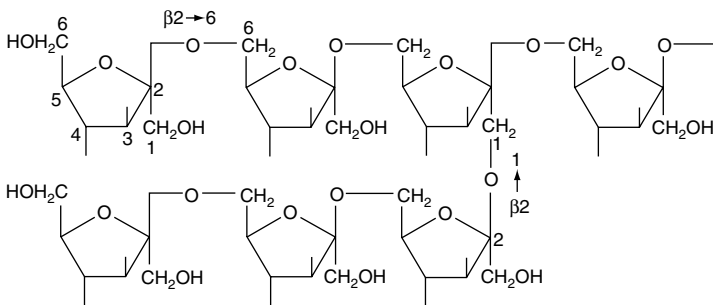


Fig. 15.7 Levan. Levan is a fructan (from fructose). It consists of a $\beta 2 \rightarrow 6$ backbone and a $\beta 2 \rightarrow 1$ crosslinks. Carbon atoms are numbered (*top left*) and vertical lines up or down from the ring indicate the OH group position. The enzyme, fructosyl transferase, is present in various oral bacteria but not *S. mutans* (Extensively modified from Fig. XI in US patent 7,528,100 B2 <http://www.bing.com/images/search?q=Levan+polysaccharide&go=&form=QBIR&qsn=&sk=#focal=92a464cd00c520daee834d27a0b2c34c&url=http%3A%2F%2Fwww.uspto.gov%2Fweb%2Fpatents%2Fpatog%2Fweek18%2F0G%2Fhtml%2F1342-1%2FUS07528100-20090505-C00021.gif>)

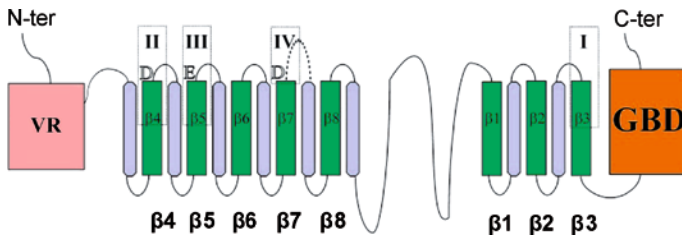


Fig. 15.8 Glucosyl transferase structure. Virtually, all carbohydrate synthesizing and degrading enzymes are structurally related (in families) because polysaccharides (glycans) are much less diverse than polypeptides. Glucosyl transferases (family 70) are related to the amylases (family 13) and the catalytic structure is a putative barrel composed of eight α -helices and eight β -strands like α -amylase (depicted in Fig 12.12a) and containing a similar catalytic site. This site allows glucosyl residues to become transiently attached to an aspartate residue corresponding to the aspartate residue in Fig. 12.11. The barrel forms from β -sheet/ α -helix sequences in the same order as they appear in the above polypeptide N-terminal to C-terminal. This order of strands and of conserved regions differs from those in salivary amylase (Fig. 12.12a). The barrel starts at α -helix₃ and ends at β -strand₃, whereas in salivary amylase, it starts at β -strand₁ and ends at α -helix₁. Domain B, between β -strand₃ and α -helix₄ of salivary α -amylase, is replaced with a large stretch of sequence of unknown function between α -helix₈ and β -strand₁. The barrel region is preceded by a long N-terminal region of variable length (Variable Region, VR) that is absent from salivary amylase. At the C-terminal end, domain C (which enhances the solubility of salivary amylase) is replaced with a Glucan Binding Domain (GBD) (Adapted from Fig. 2B in van Hijum SAFT, Kralj S, Ozimek LK, et al. (2006). Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiology and Molecular Biology Reviews*, 70(1):157–176)

The glucosyl transferases have a domain structure (Fig. 15.8) similar to α -amylase (Sect. 12.5.1). The *S. mutans* genome possesses three glucosyltransferase genes (*gtfB*, *gtfC* and *gtfD*) whose products are expressed constitutively and form the capsule at the cell surface. The GTF-I (*gtfB* gene) mostly makes the water-insoluble mutan. The GTF-S (*gtfD* gene) mostly makes the water-soluble dextran, and the GTF-SI (*gtfC* gene) makes a mixture of water-soluble short chain mutan and dextran. All three GTF proteins have two functional domains (Fig. 15.8): a large, central barrel-like domain that is catalytic and a C-terminal domain that binds to the glucan polymer (Glucan Binding Domain, GBD).

The reaction catalyzed by glucosyl transferase (Fig. 15.9a) is likewise similar to that catalyzed by α -amylase (Sect. 12.5.1). The glucosyl moiety of a sucrose molecule (Fig. 15.9b-i and c-i) is transferred to an aspartyl ester on GTF-I or GTF-S (analogous to Asp 197 in saliva amylase; Fig. 12.11) and a fructose residue is released (Fig. 15.9b-ii and c-ii). The release of fructose frees the enzyme to bind to a second sucrose molecule whose glucosyl residue displaces the first glucosyl residue on the activating aspartate residue. The displaced, first glucosyl moiety immediately forms a glycoside bond with the 3-OH group of the new aspartate-bound glucosyl residue (in GTF-I) or the 6-OH group of that residue (in GTF-S). The glucan chain extends at the C3 or C6 end by one glucose residue (Fig. 15.9c-iii).

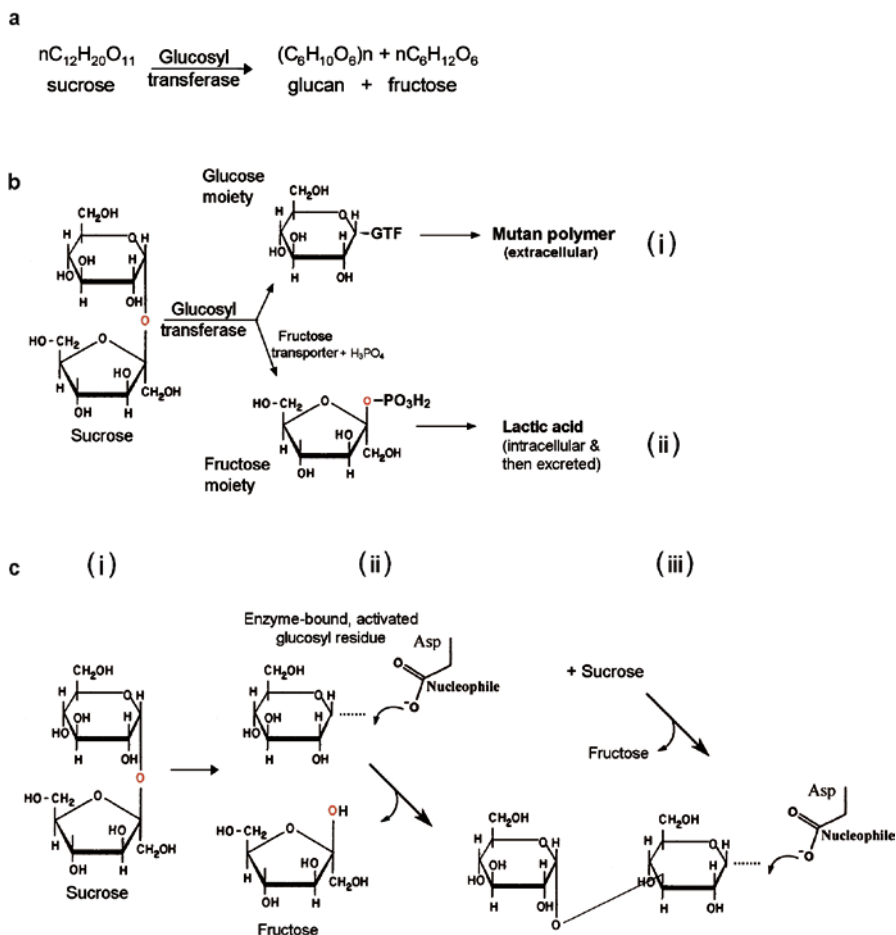


Fig. 15.9 Synthesis of mutan from sucrose by *S. mutans*. **(a)** Reaction of sucrose with glucosyl transferase (see text). **(b)** Fates of the products. (i) The sucrose is hydrolyzed at its glycoside bond (red O atom also shown in c-i). The glucose moiety b-i is briefly enzyme bound as indicated in [c(ii)] and polymerized to mutan by the enzyme interacting at the C1 and C3 positions. The fructose is transferred intracellularly and metabolized to lactose which is secreted. The fructose metabolism provides energy. **(c)** Glucosyl transferase mode of action (see text)

This process resembles the synthesis of starch and glycogen. Instead of nucleoside diphosphate glucose (UDP-glucose) being hydrolyzed to release UDP as the glucosyl residues form starch or glycogen, sucrose releases fructose and the enzyme-bound activated glucosyl residues form a mutan or dextran. The process is repeated until stopped by lack of sucrose. The polymer may then be transferred to water or to make a branch: i.e., to a C₆-OH group on an adjacent mutan chain; or to a C₃- or C₄-OH group on an adjacent dextran chain.

15.2.2.

Sources of Bacterial Lactic Acid in Caries

The fructose released during this process (Fig. 15.9b-ii) binds to a glycan phosphotransferase system (PTS) of enzymes on the cell surface. This binding activates an enzyme on the cytosolic side of the PTS to attach a phosphate residue from phosphoenolpyruvate in the cytosol to a histidine residue of a PTS protein on the cytosolic side of the inner membrane. This activates the PTS enzyme system to transfer fructose 6-phosphate to the cytosol where it is phosphorylated and metabolized to lactate by glycolysis (Fig. 1.7). The PTS returns to its original conformation and another fructose molecule attaches and is transferred. The lactic acid is excreted, but trapped at the tooth surface by the glucan capsule.

Salivary α -amylase is a protein that contributes to the enamel pellicle (Sect. 12.1.3). More importantly, it attaches bacteria, especially streptococci, to teeth surfaces. Thus, following a meal rich in carbohydrates, amylopectin, amylase, and glycogen are digested to *maltose* at the surface of many oral bacteria. The maltose is taken into the cytosol by a phosphoenolpyruvate transporter homologous to the fructose transporter of *S. mutans*. Within these bacteria, the maltose is digested to two molecules of glucose 6-phosphate and metabolized to lactic acid. Thus, twice as much acid is produced per mole maltose than per mole sucrose and it contributes to tooth demineralization even if less sucrose is consumed.

Actinomyces spp. and the various viridans species of streptococci are more common in the oral cavity than the mutans species of streptococci. They bind to teeth surface-adherent salivary proteins in the absence of sucrose (Sect. 12.6.1) and make glucans and fructans that enhance mutans streptococcal adherence and acid production in the presence of sucrose. *S. mutans* and other oral streptococci express *glucan binding proteins* that stabilize the *S. mutans* capsule in vitro and enhance its adherence to these other bacterial glucans on teeth surfaces in vivo. Fructan and dextran polymers that are not bound to mutan are digested by bacterial enzymes, *dextranase* and *fructanase*. As mutan cannot be digested (Sect. 15.2.1), its stability promotes lactic acid retention and caries.

15.2.3.

Dentinal (Advanced) Dental Caries

Because only *S. mutans* survives in acid (below pH 5.5), repeated sucrose intake tends to increase the *S. mutans* colonization of sheltered “stagnation” regions where cavities mostly develop. As the region becomes more acidic, the developing cavity promotes the growth of yet more acid-tolerant bacteria, the *Lactobacillus* spp., especially *Lactobacillus casei* (*L. casei*) which can survive for a few hours in dilute hydrochloric acid (pH 1). Lactobacilli are short fat rods similar in shape to *E. corrodens* (Fig. 13.3d), but larger and gram positive like *S. mutans* (blue staining) instead of gram negative (red staining). Lactobacilli colonization is associated with the expansion of a cavity from enamel into dentin.

Outside a carious cavity, *S. mutans* accounts for less than 1% of the microbiota on teeth surfaces, and lactobacilli for less than 0.1%. Within a developing cavity, *S. mutans* often accounts for 5% or more of the microbiota. Once a cavity is established, the local pH often falls below 4.0, which stops the growth of *S. mutans* and all other oral bacteria except *L. casei*. The *L. casei* grows on the monosaccharide residues of proteoglycans at the enamel–dental junction and in the non-mineralized portions of dentinal sheaths and tubules. *S. mutans* therefore initiates enamel dissolution and lactobacilli dissolve dentin. The lactobacilli excrete lactic acid which dissolves dentinal hydroxyapatite, releasing collagen. Asaccharolytic bacteria follow the lactobacilli, infect the pulp and spread to the bone at the apex of the infected tooth.

S. mutans possesses glucosyltransferases that make a metabolically stable mutan capsule from the glucosyl moiety of sucrose. The fructose moiety of the sucrose is metabolized to lactic acid. Other bacteria possess a fructosyltransferase that transfers the fructosyl moiety to a fructan and metabolizes the glucosyl moiety to lactate. Mutan interacts with dextrans and fructans through glucan binding proteins. The result is a sticky matrix that retains lactic acid at the tooth surface. Many oral bacteria bind salivary amylase, which converts starch to maltose. Maltose is transported into the cytosol and metabolized to lactate. The transport of maltose, fructose, or glucose into the cytosol involves a group of enzymes that transfer the phosphate residue of cytosolic phosphoenolpyruvate to the sugar, the phosphoenolpyruvate transport (PTS) system. As the region becomes more acidic, the developing cavity promotes greater colonization by *S. mutans* and even more acid-tolerant bacteria, *Lactobacillus* spp. The latter expand the cavity into dentin. Asaccharolytic bacteria of the oral microbiota become protected from the acid by metabolizing the exposed dentinal proteins in the demineralized dentin. These bacteria induce painful inflammation and abscesses within the pulp and periapical regions of a tooth affected with untreated late-stage dental caries.

15.3.1.

Variation in Individual Human Caries Experience

As in rodents and other animals, there is much individual variation in human caries experience. In the Vipeholm study, 25% of subjects taking the sticky candies did not develop any cavities over 6 years, whereas a few cavities appeared in control subjects who received a diet that contained little carbohydrate and no refined carbohydrate. A few cavities also appeared in children of the Hopewood House study who received a similar diet. Within 74 junior and senior dental students attending the College of Dentistry at the University of Oklahoma in 1985 (mean age 26 years), the mean DMFT was 8.4 with a variability of 40% about the mean (Fig. 15.10). Two had only one tooth affected and two others had, respectively 15 and 16 teeth affected. The variation is due to differences in microbiota, dietary carbohydrate intake, saliva flow, fluoride exposure, and acquired immunity (Table 15.1).

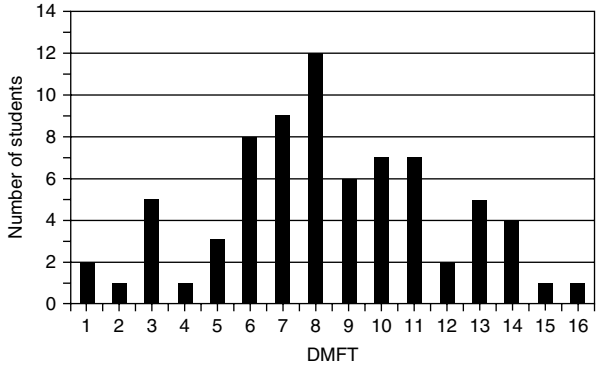


Fig. 15.10 Distribution of caries in a dental student population. The number of decayed, missing, and filled teeth due to caries (excluding third molars) was obtained for 74 junior and senior dental students attending the University of Oklahoma College of Dentistry in 1985 (M. Levine: Unpublished data)

Table 15.1 Factors determining dental caries severity

1.	Differences in the gram-positive composition of supragingival microbial biofilms
2.	Dietary carbohydrate ingestion, especially its sucrose content, and also its physical consistency and frequency of ingestion
3.	Saliva access to teeth surfaces and its flow rate
4.	Fluoride in the drinking water and toothpastes
5.	Antibodies to <i>Streptococcus mutans</i> in blood and the oral cavity

Saliva flow and its acquired immunity proteins are discussed in Chap. 12, Sects. 12.1.3. and 12.1.4. Fluoride is discussed in Chap. 16. It should be noted that despite considerable efforts, an association between sucrose intake and caries experience (Fig. 15.2) was only established for large populations, not individuals.

15.3.2. Bacterial Causes of the Variation in Caries Susceptibility

The microbiota of the oral cavity forms within a few months of birth and is stable thereafter. The types of bacteria present in the biofilm adhering to teeth, and therefore the extent of caries development depends at least in part on that microbiota. Individual differences in microbial composition cause individual variation in caries development.

In addition to variations in a biofilm's polymer content related to sucrose consumption, bacteria that metabolize salivary urea or dietary arginine also affect biofilm pH. The enzyme urease converts salivary urea to ammonia and carbon dioxide (Sect. 12.1.2),

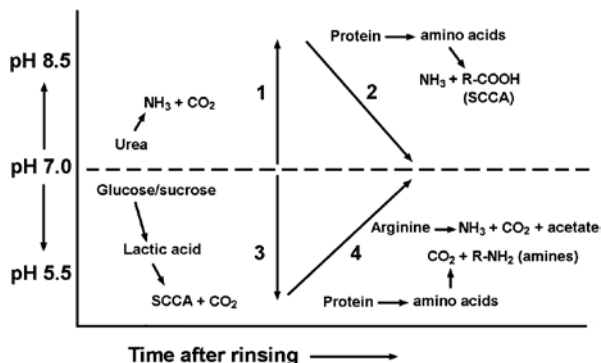


Fig. 15.11 Factors affecting pH after a 10% glucose or sucrose mouth rinse. Dashed line indicates pH 7. Vectors 1 and 4 increase the pH; vectors 2 and 3 decrease the pH. The short-chain carboxylic acids (SCCA) may be produced from amino acids along with ammonia, or from the catabolism of lactic acid (see text and also Fig. 1.8). (From Fig. 9 in Kleinberg I, 2002. "A mixed-bacterial ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to *Streptococcus mutans* and the specific-plaque hypothesis." Crit Rev Oral Biol Med 13:108–125)

promoting a faster pH rise after consuming glucose or sucrose (Fig. 15.11). Arginase converts dietary arginine to ornithine and urea which is acted on by urease. The ornithine is ultimately converted to ammonia and acetate.

The short-chain carboxylic acids (SCCA) produced by asaccharolytic metabolism (Sect. 1.3.2) are less acidic than lactate, and more common at the base of a gingival sulcus or pocket than coronally. Correspondingly, the pH is alkaline in this region (Sect. 1.3.2) because of the greater ammonia production, and a carious cavity does not normally extend into the gingival sulcus. This observation gave rise to a clinical rule called, "extension prevention," which is no longer applied. Extension beneath the sulcus predisposes to periodontitis and a root surface exposed in the oral cavity where caries can redevelop.

Counts of the number of *S. mutans* or *L. casei* are a poor measure of caries susceptibility because as noted above, they cannot account for ammonia production or differences in glycan production by other bacteria in the biofilms. Acid production after a 10% sucrose or glucose rinse after a 6 h or longer fast is a better measure of caries susceptibility, because it represents the balanced outcome of the factors that promote or prevent caries. Because this method requires time and skill, it is difficult to apply to many subjects. A simpler method of measuring caries susceptibility is past caries experience and age. The more teeth affected by caries in a child or adolescent, the greater is the likelihood that a new cavity will develop and, therefore, the greater is the need for preventive measures. Unfortunately, this measure is completely uninformative about what factors are present to promote or prevent caries.

15.3.3.

Saliva Causes of Caries Susceptibility

A major factor mediating resistance to caries is the flow of saliva over the teeth and its bicarbonate content. Thus, a low salivary bicarbonate content, or restriction of flow due to excessive mouth breathing or salivary gland disease causes a steeper Stephan curve. The pH takes longer to return to resting levels and associates clinically with severe caries. In addition, the shape and spacing of the teeth affects saliva access, and individuals who have large, overcrowded teeth that are in tight contact with each other develop more cavities than individuals with small, well-spaced teeth. Orthodontic treatment prevents caries by alleviating the crowding, which enhances the access of saliva to teeth surfaces. Orthodontic therapy is an important preventive measure to ensure that as much saliva circulates over the tooth surface as possible.

A controversial area of salivary influence on caries involves the acidic proline-rich proteins (Sect. 12.6.1). There are five such proteins, Pa, Pif, Db, Pr1, and Pr2. Pr1 and Pr2 are encoded at the *PRH2* locus, whereas the first three are encoded at the *PRH1* locus. These two loci encode all possible alleles of the salivary acidic PRPs in Caucasians (Fig. 15.12). Knowledge of the human genome sequence has permitted the genes encoding each of the respective PRP proteins to be amplified by polymerase chain reaction (PCR). Because protein Db is larger than any other (allelic) protein encoded by the *PRH1* or *PRH2* locus, the PCR product indicates the presence of Db in genomic DNA, and therefore in saliva.

In 3 – 5 year-old children of low socio-economic status, Caucasian children not expressing Db exhibit twice the severity of caries than do African-American children who have a

Genetic differences in caries susceptibility may be due to different combinations of acidic proline-rich proteins in different racial groups

<i>PRH1</i> locus ^a		<i>PRH2</i> locus ^a	
Chromosome-12A Protein	Chromosome-12B Protein	Chromosome-12A Protein	Chromosome-12B Protein
Db ^b	Db	PRP-1	PRP-1
Db	Pa	PRP-1	PRP-2
Db	Pif	PRP-2	PRP-2
Pa	Pa		
Pa	Pif		
Pif	Pif		

^aTotal = 6 *PRH1* allelic combinations X 3 *PRH2* combinations = 18

^bDb inhibits caries in young children

Fig. 15.12 The PRP protein alleles. The three proteins encoded by the *PRH1* locus are on the left two columns and the two proteins by the *PRH2* locus on the right two columns. Because this locus is expressed from both parental genes (A and B), there are six possible protein (allelic) combinations of Pa, Pif, and Db and three possible combinations of Pr1 and Pr2. This gives a total of 18 combinations for each of the six combinations encoded by the *PRH1* locus is paired with one of the three combinations encoded by the *PRH2* locus

2.5-times greater frequency of Db in saliva. Yet at age 12, the Caucasian children without Db have less caries experience than Caucasian children with Db. This latter finding was associated with greater *S. mutans* adhesion to Db, which is part of the acquired pellicle (Sect. 12.6.1). The different mixtures of acidic proline-rich proteins in African-Americans may inhibit this Db-associated process, protecting them from caries. Because 60% of cavities occur in only 20% of individuals, identifying caries-susceptible individuals early improves the targeting of procedures to prevent caries before it is severe. Comparing Db within the full complements of acidic proline-rich proteins in young children of African-American and Caucasian descent may better identify sub-populations who are biologically more susceptible to caries.

15.3.4. Caries Immunity and Susceptibility

Immunizing caries-susceptible rats and mice with *S. mutans* prevented caries from developing when these animals were fed a caries-promoting diet (see Sect. 15.1.4). Subsequently, the rodents were also protected from caries if they were immunized with the glucosyltransferases purified from *S. mutans*.

Similar systemic immunization experiments were carried out in monkeys. Monkeys fed candies and cookies and given water containing 2% sucrose developed many cavities after 3–4 years unless immunized with whole cells of *S. mutans*. Various purified protein antigens given by subcutaneous vaccination induced a blood plasma immunoglobulin response that protected monkeys from caries, whereas the glucosyl transferases protected only if vaccination methods that promoted salivary IgA immunity Sect. 12.1.4 were used.

The major protective antigen of *S. mutans* was a cell-surface adhesin named AgI/II (Fig. 15.13). Antigen I/II attaches *S. mutans* to teeth surfaces by binding to salivary agglutinin (Sect. 12.6.2). Variants of antigen I/II are present in many streptococci, not just *S. mutans*. Attachment to salivary agglutinin may explain the predominance of viridans streptococci in the initial biofilms that form on the acquired pellicle in experimental human gingivitis (Sect. 12.1.5). Antigen I was purified by 1984 to vaccinate children against caries, unfortunately coinciding with reports that this antigen cross-reacted with human heart proteins. It was subsequently found that an impurity not present in the purified antigen had induced the heart cross-reactivity. Nevertheless, a fear of problems led to the vaccination proposal being dropped. Indeed, most individuals do not require immunization because of almost universal access to fluoridated drinking water and toothpastes (Sect. 16.2.1). In regions such as the US, Western Europe, and Australia/New Zealand, no more than 20% of the population suffers from severe caries.

Severe caries tends to occur in rural or economically disadvantaged children and can lead to oral and/or health problems later in life. These children usually have no access to fluoridated water or toothpastes, let alone regular dental care. Vaccinating such at-risk populations with antigen I/II or glucosyltransferase could provide effective caries protection at little risk. Unfortunately, there is currently no biologically based susceptibility test to diagnose a child at risk for severe caries. Identifying which acidic PRP alleles are present in genomic DNA along with measurements of salivary agglutinin levels in saliva might provide a suitable test.

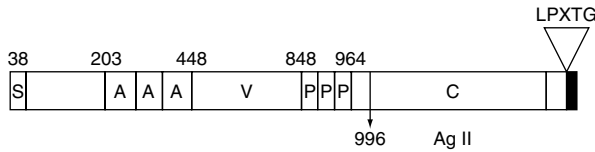


Fig. 15.13 Major adhesion antigen of *S. mutans*. The amino acid sequence of antigen I/II (1,561 residues; mol. wt. 185,000 Da) begins with a putative signal peptide (S). The N-terminal region (residues 157–448) is an alanine-rich domain whose three subdomains are made up of a 7-amino acid tandem repeating sequence that possesses at least one alanine residue (A). The tandem repeats begin at residue 203 and end at residue 448, each set of repeats being 82 residues. This domain likely forms a set of juxtaposed α -helices. Following a short linker sequence, the third domain (461–834) is a lectin-like variable region (V) that may bind glycan. A fourth domain has three homologous proline-rich domains (residues 848–964) that favor an extended conformation. The remaining sequence is a C-terminal domain (C) within which residue 1535 near the C-terminus (within the *black region*) is a conserved threonine residue (not shown) whose hydroxyl group is covalently bonded by an amide link to cell wall peptidoglycan (Sect. 1.4.1). At the N-terminal end of this region is a strongly conserved sequence (LPXTG) that is a cleavage site for amidases which release the antigen from the cell wall. X stands for any amino acid. It varies between genus and species and is always N-terminal on the fragment that remains covalently attached to murein. Antigen II is antigen I truncated at residue 996 and has therefore lost all the upstream N-terminal regions (Derived from Fig. 1 of Younson J and Kelly C (2004) “The rational design of an anti-caries peptide against *Streptococcus mutans*.” Molecular Diversity 8:121–126. Domain and repeating sequences taken from UniProtKB/Swiss-Prot P11657 (PAC_STRMU) modified September 22, 2009 (Version 74))

Caries experience depends on the microbial composition of biofilms, amount of sucrose and refined carbohydrate in the diet, saliva flow, fluoride in drinking water and in toothpastes, and immunity to *S. mutans*. Biofilms are a mixture of symbiotic bacteria, entrapped in an adhesive capsule. Individual differences in microbial composition cause variations in polymer content. The extent to which a microbiota utilizes salivary urea or dietary arginine to make ammonia affects biofilm pH and caries experience. Diseased salivary glands, excessive breathing through the mouth instead of the nose, and teeth that are tightly squeezed together impede access of saliva and promote caries. Greater biofilm colonization and caries may be promoted by high levels of salivary agglutinin in subjects whose saliva also secretes a specific acidic proline-rich protein allele, Db. Nasal or oral spray immunization with glucosyl transferase induces salivary IgA that protects rodents from caries despite a cariogenic diet. Subcutaneous immunization with mutans adhesion antigen I protects primates by reducing *S. mutans* colonization of the oral cavity. Severe caries tends to occur in rural or economically disadvantaged children and it can lead to oral and/or health problems later in life. Vaccinating such children seems an attractive therapy that could be explored further.

This chapter describes how individuals with severe enamel fluorosis (mottled tooth enamel) became associated with fluoride in the public water supply and protection from dental caries. A comparison of caries experience with the fluoride content of public water supplies and enamel fluorosis in adolescents indicated that 1 μg fluoride/mL (1 part/million) in the water provides caries protection with minimal enamel fluorosis (sect. 1). One mechanism is the spontaneous isomorphic replacement of apatite's hydroxide anions with fluoride, which reduces enamel solubility. A second is fluoride-mediated inhibition of enolase, which retards bacterial acid production at teeth surfaces. These findings led to the use of fluoride in toothpastes, which provides better protection from caries at tooth surfaces than water fluoridation alone (sect. 2). The chapter concludes with a discussion of potentially harmful effects of fluoride ingestion (sect. 3).

16.1.1.

Properties of Fluorine and Fluoride

The element fluorine has nine protons, ten neutrons, and nine electrons, giving a mass of 19 Da. Fluorine is the smallest halide element (Sect. 1.1.5) and it forms a gas that is so electron deficient that it has all reacted with metals and only its ions (F^{-1}) are found on earth. Fluoride and hydroxide ions have almost the same mass, 19 Da and 17 Da respectively. Because fluoride is more electronegative, it replaces the hydroxide ion on crystals without altering their overall structure. This type of alteration is referred to as an *isomorphous* ion replacement.

The fluoride ion content of solutions is measured using a fluoride electrode. The sensor is a crystal of lanthanum trifluoride containing small amounts of europium difluoride (Sect. 1.1.5). Because lanthanum forms a tri-fluoride lattice, but europium forms di- or tri-fluoride lattices, vacancies of fluoride ions develop in a mixed lanthanum-europium crystal. If a solid electrochemical cell is constructed from this mixed crystal, differences in the transference of charge are exclusively due to differences in fluoride availability, provided the samples are acidic. Hydroxide ions interfere with fluoride transfer. The cell is standardized by inserting it into known concentrations of fluoride solutions and the voltage measured. A graph of voltage against fluoride concentration then allows the fluoride content of an unknown solution to be calculated.

16.1.2.

How Mottled Enamel Relates to Fluoride in the Water Supply

The studies that identified fluoride in the water supply eventually led to its use in caries prevention. These studies were mostly due to the extraordinary abilities of one man, Frederick Sumner McKay (Fig. 16.1). McKay graduated in dentistry in Boston but in 1901, he moved to Colorado Springs because of health problems. There, mottled enamel was common and he soon became acquainted with this problem because many of his patients were seeking treatment for unsightly teeth due to mottling, not dental caries as in Boston. In 1908, McKay sent samples of mottled teeth to G. V. Black, Dean of the Northwestern University Dental School of Chicago, for advice. Beginning in 1916, he and Black published a number of reports in *The Dental Cosmos*, which later merged with the *Journal of the American Dental Association*. McKay outlined the geographic boundaries of the areas where mottled enamel was found, determined the percentage of afflicted individuals, and compiled evidence that pointed to the water supply as a cause. Black detailed histological studies that compared the enamel structure of mottled and normal teeth. Between 1925 and 1931, similar changes were described in the teeth of rats fed sodium fluoride in their diet, but these changes were not compared with Black's description of mottled human enamel.

At the same time, McKay was informed of an outbreak of mottled enamel in children who lived in Bauxite, a town near Colorado Springs. Bauxite was named for large deposits of bauxite, which the Aluminum Company of America (ALCOA) mined there. Children who drank spring- or shallow-well water had normal teeth, but children drinking deep-well water had severe enamel mottling. In 1931, ALCOA's chief chemist,



Fig. 16.1 Dr Frederick Sumner McKay (From "Obituary: Frederick Sumner McKay." (1960) *American Journal of Orthodontics* 46(9): 695–699)

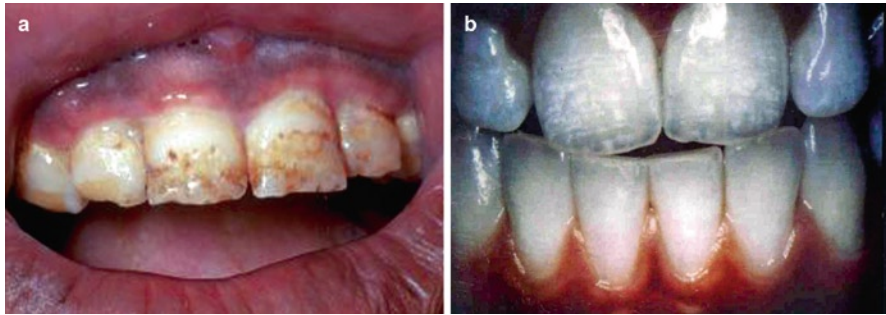


Fig. 16.2 Mottled enamel. (a) Severe mottling. Note the wear at the coronal edge of the central incisors, the brown color, and holes on the coronal half of the crowns and the opaque white color interspersed with brown spots on the apical half (Figure downloaded from The Free Dictionary <http://img.tfd.com/mosby/thumbs/500087-fx12.jpg>). (b) Mild mottling. Note the speckles of opaque white color on the labial surface of the central incisors. Other teeth may be similarly affected (Figure is a gift from Dr. Kenneth W. Stephens, University of Glasgow College of Dentistry)

Dr. Churchill, read McKay's reports and ordered a test that identified trace elements in water using a new quartz spectroscope. The instrument identified fluoride in the deep wells only. McKay then obtained samples of drinking water from the many communities where he knew mottled enamel was found and Churchill confirmed the presence of fluoride. The association of fluoride with mottled enamel was published in the *Scientific American* in 1931.

Dr. H. Trendley Dean, a dental officer of the U.S. Public Health Service, advanced McKay's findings, identifying various amounts of naturally occurring fluoride in the public water supplies from certain parts of the US (Fig. 16.3). By 1936, Dean had found that fluoride levels of up to 1.0 part per million (1 ppm = 1 mg/L) in the drinking water did not cause mottling, or obvious dental fluorosis. More importantly, he also reported a correlation between the fluoride content of the water and a reduced incidence of dental decay. Community-wide studies were carried out to evaluate the effect of fluoride in the water supply on caries by adding sodium fluoride to fluoride-deficient water supplies. These studies began in Grand Rapids, Michigan, in 1945.

16.1.3.

Mottled Enamel Is Moderate to Severe Enamel Fluorosis

Fluoride is both incorporated into enamel crystals and also affects the enzymes involved in enamel formation (Sect. 16.2.2), causing mottled enamel, a severe example of enamel fluorosis. Enamel fluorosis is evident as specks or white flecks on the enamel surface (Fig. 16.2b). In 1941, the public water supply of Aurora (IL) contained 1.2 ppm of fluoride (F), but only 5% of teeth exhibited fluorosis, mostly premolars and second molars. A sensitive index of

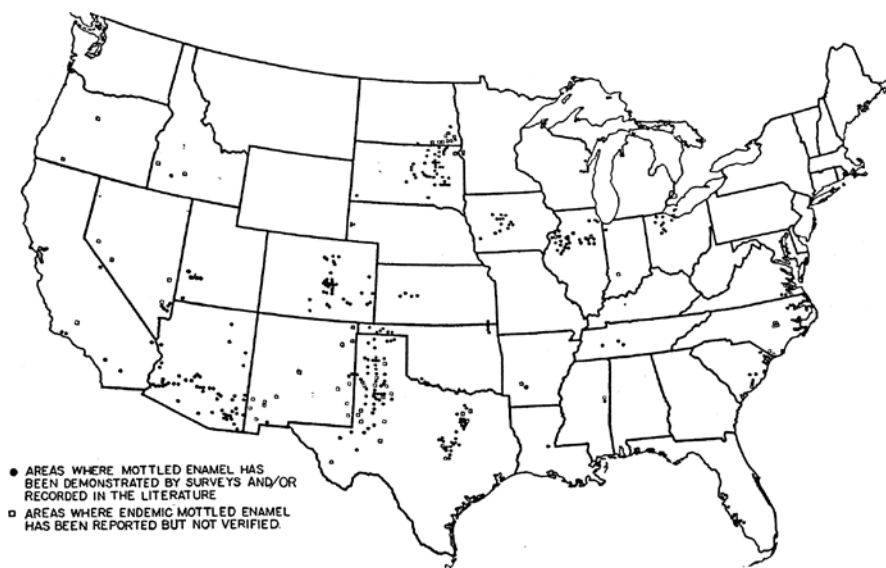


Fig. 16.3 Geographic location of mottled enamel in the US (1941) (Figure is a reproduction of Fig. 2 from Dean HT, Kitchin P, Moulton FR (eds) *Fluorine and dental health*. American Association of the Advancement of Science Publication No 19: Washington DC 1942 © AAAS 1942, pp. 6–11)

fluorosis requires children aged 12–13 in whom the premolars and second molars have erupted. The mottled enamel first drawn to McKay's attention in Colorado is moderate and severe fluorosis (Fig. 16.4).

The level of fluorosis in an individual is determined by observing the buccal surface of each tooth and classifying it as one of the following: *Normal (score 0)* Enamel presents the usual translucent, appearance. The surface is smooth, glossy, and usually of a pale creamy white color; *Questionable (score 1)* The enamel has slight aberrations from normal translucency, ranging from a few white flecks to occasional white spots; *Very mild (score 2)* Small, opaque, paper-white areas scattered over less than 25% of the tooth surface or a premolar or molar tooth showing no more than 2 mm of white opacity at the tip of the cusps; *Mild (score 3)* White opaque areas are discontinuous or a continuous white opacity affects less than 50% of the tooth surface (Central incisors in Fig. 16.1b); *Moderate (score 4)* More than 50% but not all of the enamel surface is affected with a continuous white opacity and little or no brown is present (Fig. 16.4); *Severe (score 5)*; All of the enamel surface displays a continuous white opacity or more than half of the tooth surface is stained brown and surfaces are subject to mild attrition (Figs. 16.1a and 16.4). An individual is assigned a number (0 through 5) which represents two or more teeth with the greatest severity score. The index of fluorosis is the mean of these individual scores within a community.

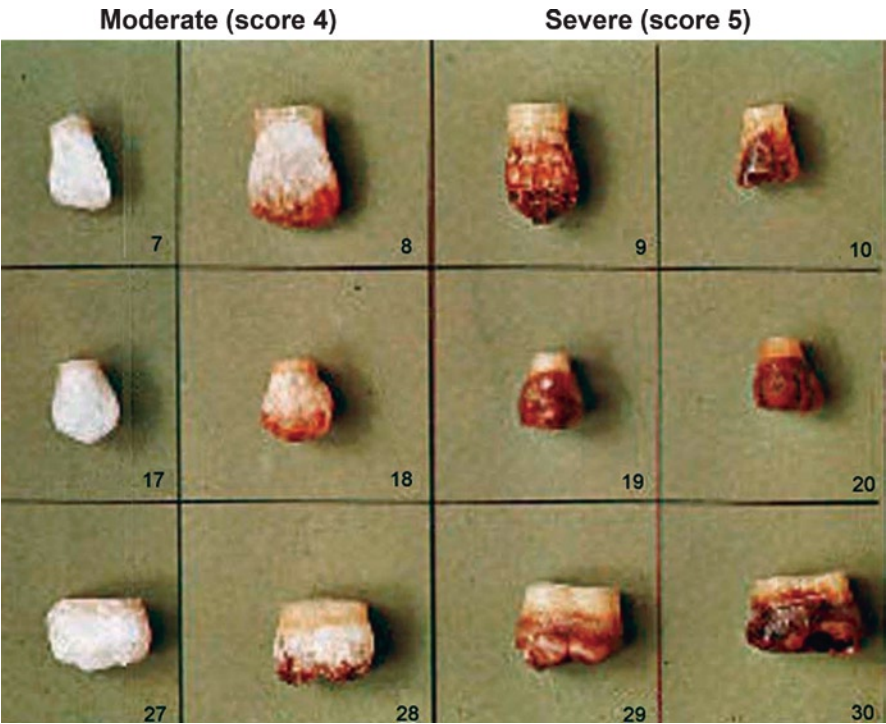


Fig. 16.4 Index of fluorosis. Figure depicts moderate to severe fluorosis of the lateral incisor, canine, and second molar teeth: index values of 4 and 5 (Copied from the University of Oklahoma Library Collection circa 1990, Source unknown)

16.1.4.
Identification of 1 ppm Fluoride in the Water as Optimal for Cavity Protection

The greater the fluoride content of the public water supply, the greater is the index of fluorosis. At less than 1 ppm fluoride in the drinking water, the index of fluorosis in the community is essentially zero (no fluorosis) and increases to severe at 8–10 ppm fluoride in the drinking water. Fig. 16.5 illustrates how the severity of fluorosis changes with amount of fluoride in the community water supply and also the average number of decayed, missing, and filled teeth (DMFT, Sect. 15.1.1). The subjects were children aged 12–14 years in various US cities and communities and their DMFT is compared in Fig. 16.5 with the ppm fluoride in their water supply.

If the water supply had no fluoride, the population displayed 6–8 DMFT. As the fluoride concentration increased to 1 ppm, the mean DMFT of the population decreased by about 50%, but the index of fluorosis remained well below 1. Increasing the fluoride above 1 ppm

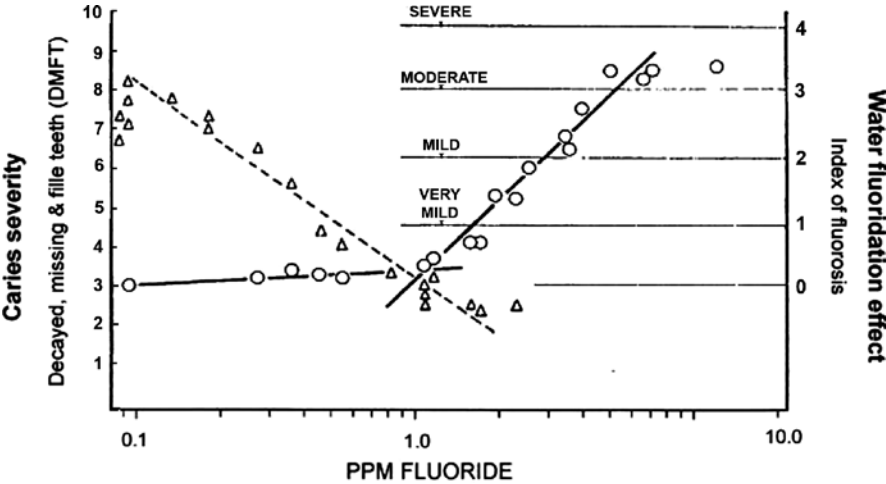


Fig. 16.5 Relationship of DMFT and index of fluorosis to water fluoride content. The left y-axis indicates the number of decayed missing and filled teeth from caries (DMFT) and the right y-axis indicates the index of fluorosis, a measure of the deleterious effect of fluoride on the enamel surface (see text). The x-axis indicates the ppm fluoride found naturally in the drinking water supply. Triangles indicate DMFT and circles indicate the fluorosis index in the same populations. The curves showing the decrease in caries and decrease in fluorosis intersect at ~1 ppm fluoride in the water supply on the x-axis (Copy of Fig. 3 from Hodge HC, Smith FA. (1954). Some public health aspects of water fluoridation. American Association of the Advancement of Science Publication No 19: Washington DC 1954 © AAAS 1954, pp. 79–109)

slightly decreased the DMFT further, but markedly increased the prevalence of fluorosis. The line of decreasing fluorosis intersects with the line of decreasing caries at just over 1 ppm fluoride as seen in the center of Fig. 16.5.

The fluoride ion can replace the hydroxide ion in a crystal without significantly altering its structure, an isomorphous ion replacement. Fluoride also affects the enzymes involved in enamel formation, causing mottled enamel, a severe example of enamel fluorosis. White opaque patches on the normally translucent enamel indicate mild fluorosis. Fluorosis is measured on a grade of 0–5 where 1 through 3 indicate an increased cover of opaque white patches on the tooth surface, and 4 and 5 indicate an increased mottling. The two worst affected teeth make up an individual’s score. The community’s index of fluoridation is the mean score for all individuals. As the natural or artificial fluoride concentration of the water supply increases to 1 ppm, the mean number of cavities in 10–12 year-old children decreases from 7 to 3. Above 1 ppm fluoride, caries does not decrease much more, but the index of fluorosis increases markedly. This is the reason why public water supplies are fluoridated to only 1 ppm and not more or less.

16.2.1. Mechanisms of Fluoride Protection from Caries

Dental caries occurs when bacterial acids dissolve the hydroxyapatite crystals of enamel and produce amorphous sodium monohydrogen phosphate from hydroxyapatite by reversing the solid-state reactions shown in Fig 16.6. This process occurs when bacteria in a biofilm produce lactic acid by saccharolytic fermentation (Sect. 15.1.3). Protons (H^+) diffuse into the crystal where they react with hydroxyl groups in hydroxyapatite and change the crystal to an amorphous calcium monohydrogen phosphate solid that slowly dissolves (Fig 16.6b). An increased lactic acid concentration ($pH < 6.2$) makes the dissolution proceed faster by converting calcium monohydrogen phosphate to calcium dihydrogen phosphate which is 10 times more soluble. The critical pH for apatite dissolution is pH 6.2. Apatite dissolution is slow above pH 6.2 and fast below pH 6.2. Thus, after a 10% sucrose rinse, subjects with severe caries exhibit a pH fall that is further and longer below 6.2 than subjects with mild caries (Fig. 15.3).

The presence of even one fluoride ion in the crystal slows the transformation to amorphous calcium monohydrogen phosphate. Thus, in the presence of fluoride (e.g., after using fluoridated toothpastes), fluoroapatite forms at the tooth surface and reduces the rate of caries development. The increased fluoride concentration at the tooth surface also inhibits lactate production. These observations explain why cleaning the teeth with fluoridated toothpaste prevent caries. Cleaning the teeth exposes the apatite at the enamel surface. In the absence of fluoride, there is no protection because the biofilm forms within a few

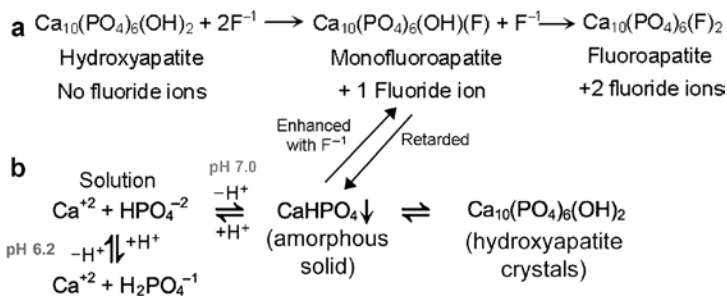


Fig. 16.6 Hydroxyapatite and fluoroapatite: formation and dissolution. **(a)** Hydroxyapatite is transformed to fluoroapatite by isomorphous replacement. Fluoride ions diffuse into a hydroxyapatite crystal where they replace the hydroxide ions. **(b)** Fluoroapatite cannot dissolve as easily as hydroxyapatite. Right to left shows the solid-state rearrangement of hydroxyapatite to calcium monohydrogen phosphate, free calcium ions, and monohydrogen phosphate. The latter becomes mostly dihydrogen phosphate above pH 6.2. Arrows between (b) and (a) indicate enhanced apatite formation or slower changes to the amorphous solid if fluoride is present. Left to right shows the precipitation of calcium monohydrogen phosphate and its change to hydroxyapatite if an acid solution is made alkaline.

minutes and dissolves the apatite as before after sugars are consumed. In the presence of toothpaste containing 1,000 ppm fluoride, fluoroapatite forms on the exposed apatite and cannot dissolve as rapidly in the acid after sugars are consumed. Beyond this, fluoridated water provides protection independently of toothbrushing by increasing the fluoride concentration within enamel as it develops.

Before fluoride was added to toothpastes (1950–1970), fluoridated water provided considerable protection from caries, but now that virtually all toothpastes are fluoridated, some have questioned the need for water fluoridation. Yet carefully controlled studies in Scotland, where public pressure to remove fluoride from the water supply has been extremely strong, indicate a marked increase in caries despite the continued use of fluoridated toothpaste (see Sect. 16.3.2). Fluoride toothpaste-mediated protection from caries is dependent on oral hygiene efficacy, whereas protection from caries by fluoride in the water supply appears independent of oral hygiene. Protection from caries by artificial fluoridation of water supplies and fluoridated toothpaste is independent and additive.

In the USA, water fluoridation became widely available after 1955 and fluoridated toothpastes after 1975. In 1954, few locations had naturally or artificially fluoridated drinking water and fluoridated toothpastes did not exist and the mean DMFT in US 12–13-year-old children was about 7. By 2004, according to the National Health & Nutrition Examination Survey, most locations had fluoridated water and fluoridated toothpaste and the mean DMFT in 11–15-year-old children had fallen from 8.0 in the mid-1950s to less than 2.0.

16.2.2.

How Fluoride Protects from Caries

The first and primary protective effect of fluoride is due to its strong, spontaneous reaction with metal ions. Biologically, the most important of these ions is the calcium ion, large amounts of which interact with phosphate to form bones and teeth. Studies show that fluoride reduces apatite solubility in acids by an *isomorphic replacement* of hydroxide ions with fluoride ions to form *fluoro-hydroxyapatite* and *difluoro-apatite* (Fig. 16.6a).

Apatites must undergo a solid-state transition to amorphous calcium phosphate before they can dissolve and the spontaneous replacement of hydroxide with fluoride ions slows the rate at which this transition occurs (Fig. 16.6b). Conversely, as an acid environment becomes more alkaline, fluoride ions promote the precipitation and crystallization of amorphous calcium monohydrogen phosphate/calcium fluoride into fluoro- and difluoro-apatites faster than amorphous calcium phosphate would crystallize into hydroxyapatite. Thus, fluoride ions have two effects on enamel that protect from caries: they slow enamel dissolution in lactic acid and promote its re-precipitation and crystallization when the lactic acid is neutralized.

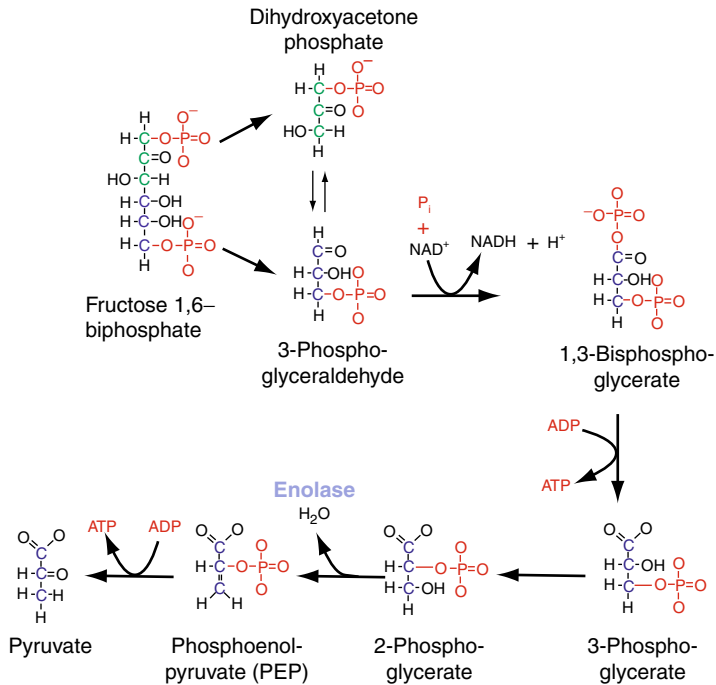


Fig. 16.7 Glycolysis showing enolase. Saccharides are processed intracellularly into UDP-sugars and metabolized to fructose 6-phosphate and then 1,6-bisphosphate if required for energy. The various steps preceding fructose 1,6-bisphosphate formation are omitted in this figure (Wikipedia public domain image slightly modified. Accessed at: <http://commons.wikimedia.org/wiki/File:GlycolysisPathway.svg>). This figure was modified by Dr Wirsig-Weichmann

A second mechanism of protection from caries is the incorporation of fluoride into bacterial biofilms where it *inhibits enolase*. Enolase catalyzes the production of phosphoenolpyruvate, the precursor of lactate in glycolysis, from 2-phosphoglycerate during glycolysis (Fig. 16.7 – see also Fig. 1.7). In addition, oral bacterial uptake of mono- and disaccharides mostly utilizes the phosphoenolpyruvate transport system to transfer them into the cytosol (Sect. 15.2.2). Fluoride therefore inhibits not only lactic acid production, but also the phosphoenolpyruvate transport system-mediated uptake of saccharide substrates. In short, fluoride inhibits saccharolytic fermentation by many oral bacteria.

Although high levels of fluoride (<3,000 ppm) kill most bacteria, there is little evidence that common levels of fluoride (1–10 ppm) alter the types of bacterial species or their relative concentrations in biofilms. Mutans and other streptococci in the biofilm may switch to asaccharolytic fermentation (Sect. 1.3.2). A *fluoride-mediated reduction in bacterial acid production*, ensues without a detectable change in bacterial biofilm composition. Correspondingly, *fluoride has no effect on the development of gingivitis or its progression to periodontitis*.

NOTE: Studies in the 1980s determined that large doses of fluoride do not protect from osteoporosis (Sect. 10.2.3), or decrease the incidence of bone fractures. It appears that increased fluoride in the diet inhibits osteoblast activity more than osteoclast activity; women on fluoride supplements suffer from more bone fractures, not less. Fluoride therapy for osteoporosis was popular in the 1980s, but the reports published after 1990 reduced enthusiasm for this treatment and it is not now recommended for post-menopausal bone loss.

In the USA, water fluoridation became widely available after 1955 and fluoridated toothpastes after 1975 and caries in adolescent children has decreased by 66%. The effects of fluoride on caries are topical from the surface to the interior. Water fluoridation ensures small amounts of fluoride throughout a tooth and fluoridated toothpaste enhances the fluoride concentration at the tooth surface. Protection from caries by artificial fluoridation of water supplies and fluoridated toothpaste is cumulative. Investigations as to how fluoridation protects from caries has identified three mechanisms of caries protection: (1) inhibition of demineralization, (2) enhancement of remineralization, and (3) inhibition of bacterial enolase activity reducing lactate production from ingested carbohydrates. Fluoride has little effect on bacterial growth, and gives no direct protection from gingivitis, periodontitis, or osteoporosis

16.3.1. Systemic Effects of Fluoride

At levels of 1 ppm, fluoride shows no skeletal or other poisonous effects. Children who drink 5–10 ppm fluoridated water develop enamel fluorosis and mottled enamel because that is when the permanent teeth are developing. Adolescents or adults who start drinking 5–10 ppm fluoridated water show no signs of enamel fluorosis and no other deleterious health effects. Fluoride ions are excreted rapidly from the body and individual cells excrete about 30% of the blood plasma concentration. In fact, acute deleterious effects require very high doses of fluoride > 1,000 ppm (>1 mg/mL) in the water or air (as occurred near Fort William, Scotland). Chronic (persistent) deleterious effects likely require a daily exposure to <10 ppm for some months and primarily show up in children and young adults as mottled enamel and skeletal defects. Reports of fluorosis being associated with an increased cancer risk in humans are probably due to high amounts of heavy metals that associate with an increased fluoride content of the water supply. Indeed, the need to purify fluoride from poisonous metals likely requires more careful oversight than is currently practiced before adding it to public water supplies in the USA and elsewhere.

In children, enamel mottling appears at >1 ppm fluoride in the water supply and bone mineralization is affected at >10 ppm. In rats exposed to >10 ppm fluoride (>0.5 μ M), radiolabeled amelogenin matrix fails to calcify, despite fluoride not affecting enamelysin or EMSP1 proteolysis of amelogenin in vitro. *Amelogenin binds better to fluoridated, carbonated hydroxyapatite* than to the non-fluoridated form, perhaps inhibiting protease binding or cleavage in vivo. If so, the serine-phosphorylated tyrosine rich peptide (TRAP peptide) remains on the large amelogenin nanospheres (Fig. 9.12; Chap. 9). Enamel

ribbons cannot calcify; enamel cannot mature and mottling results. In mottled enamel, the lack of calcified enamel rods leave the enamel with holes and the retention of amelogenin nanospheres give it a brown color (Fig. 16.1a). In mild enamel fluorosis, these defects are less severe, resulting only in opaque white regions (Fig. 16.1b).

The fluoride-mediated *inhibition of enolase* in the *glycolysis pathway* was discussed in relation to bacterial acid production (Sect. 16.2.2).

16.3.2. Fluoride Toxicity

The extraction of aluminum from bauxite, a naturally occurring mineral, causes fluoride-rich particles and fluoride gas to be released into the environment. Because it utilizes lots of electricity, the process is carried out in regions where waterfalls abound. The postindustrial revolution greatly increased the need for aluminum and a major processing plant developed in the Fort William area and continues there today. Fort William is a port on the mainland opposite the Isle of Skye to the west. To the northeast of Fort William is a long, narrow valley. In the 1940s, the plant expanded and uncontrolled fluoride emissions blew up this valley due to the prevailing southwesterly winds. The dust settled on the pasture where its high fluoride content poisoned cattle in the area. The factory was made to control its fluoride emissions, but fluoride became entrenched in the Scottish political psyche as a dangerous poison and adding it to water was vigorously opposed as potentially dangerous. As a result, many Scottish cities do not have fluoridated water despite the dental caries rate being among the highest in the world. Indeed, in the early 1990s, a moderate-sized city in Scotland, Kilmarnock actually ceased adding fluoride to their water supply.

The case for discontinuing the addition of fluoride to public water supplies has been growing in the USA, partly because of a lack of government control of the trace metal problem. The CDC has also documented a large increase in mild fluorosis now that water fluoride is combined with toothpastes. Nowadays, the ability to control fluoride dosage by tablets, toothpastes and professional administration has improved enormously in the USA. Studies on the Kilmarnock population since 1990 nevertheless indicate an increasing incidence of dental caries, indicating that self-dosage is inadequate and there is a balance to safety with disease prevention. One solution would be to reduce water fluoridation to 0.7–0.9 ppm in the USA and better enforce fluoride manufacture.

Adults exposed for long periods to greater fluoride concentrations in the aluminum and glass industries exhibit skeletal fluorosis. In the early 1980s, there were reports of crippling bone diseases caused by 2 or more ppm fluoride in the water in India and other countries. Naturally occurring fluoridated water often contains increased amounts of heavy metals and these could have caused the skeletal effects. In the USA in the early twentieth century, children drinking 2–10 ppm water exhibited enamel fluorosis and enamel mottling but no bone disease.

If the fluoride intake is maintained above 10 ppm, the inhibition of enolase (Sects. 16.2.2 and 16.3.1) inhibits both anaerobic and aerobic glycolysis. In addition, *skeletal defects* may be caused by improper collagen synthesis inhibiting proper bone formation in children. Skeletal fluorosis in children and fluoride poisoning in adults is therefore accompanied by

an *extreme lack of energy* partly due to the inhibition of enolase, but also to other effects indicated below.

The first is the inhibition of *gluconeogenesis*. Mg^{+2} ions activate fructose 1,6-bisphosphate phosphatase and convert it to phosphate and fructose 6-phosphate. The latter is a precursor of glucose and glycogen from glycogenic amino acids and of monosaccharides other than glucose. *Fluoride chelates Mg^{+2} and inhibits fructose 6-phosphate production and gluconeogenesis*. The resultant inability to make glucose and glycogen from noncarbohydrate precursors is probably a second factor contributing to the lack of energy associated with fluoride poisoning.

The second is an inhibition of protein synthesis. Fluoride interacts with magnesium ions a cofactor for binding of the large (60 S) ribosomal subunit to the small subunit tRNA/mRNA complex during translation initiation in eukaryotes. Fluoride chelates Mg^{+2} ions and *prevents the protein synthesis initiation complex from forming*. The inhibition probably occurs in association with the Mg^{+2} catalyzed GTPase-mediated hydrolysis of eukaryotic initiation factor-2 (eIF2) by the large ribosomal subunit.

A third, more controversial effect is the fluoride-mediated inhibition of enzymes that remove reactive oxygen species from the body. The enzymes catalase, peroxidase, and superoxide dismutase are primarily responsible for eliminating harmful oxygen species during respiration. The reaction of oxygen with cytochrome oxidase is only 50–70% efficient and the remaining 30–50% of oxygen is released in an incompletely reduced form known as the Reactive Oxygen Species (ROS). All these products are highly reactive in the cell despite some being relatively stable. These three enzymes prevent the more stable ROS compounds from being available to react with proteins, lipids, carbohydrates, or nucleic acids within a cell. Ascorbate is secondary to this protection (Sect. 7.4.1). *At high concentrations of fluoride in the water supply (>50 ppm) these protective enzymes, are inhibited, but there is no evidence for this at 1 ppm.*

Hydrogen peroxide is the most common, stable by-product of respiration. Each oxygen atom has received only one electron instead of two and it shares the second electron with a hydrogen atom to give H_2O_2 (hydrogen peroxide) instead of water (Fig. 16.8a). *Catalase* produces a molecule of water and a molecule of oxygen from hydrogen peroxide. A fluoride ion (F^-) competes with a peroxide ion ($^{\cdot}OOH$) for the iron atom within a heme molecule at the catalytic center of catalase. The catalase can no longer bind peroxide anions and peroxide ions from hydrogen peroxide accumulate in the tissues.

Peroxide and other partially reduced oxygen species often attach to an organic group, perhaps the amino acid side chain of a nearby protein or fatty acid, and form organic peroxides. *Peroxidase* binds to such peroxides and converts them to an alcohol by releasing the second oxygen atom that is reduced to water by 2 molecules of glutathione (Fig. 16.8b). The oxidized glutathione (GSSG) is reduced (Chap. 7, Fig. 7.8 bottom half). Peroxidase functions like catalase, but utilizes *selenium* (Se^{+2}) instead of iron at its active site. Fluoride competes for Se^{+2} , preventing the peroxide substrate from binding. Organic peroxides accumulate in the tissues and mediate spontaneous reactions that interfere with many biological processes.

Superoxide dismutase converts many other types of reactive oxygen species to oxygen gas, hydrogen peroxide, or water. This enzyme binds to these various reactive oxygen species by a molybdenum ion (Mo^{+2}) at its active center. Again, fluoride will bind to the molybdenum ion and inhibit this enzyme's activity. Reactive oxygen species accumulate in the tissues and mediate rapid, spontaneous reactions that interfere with many biological processes.

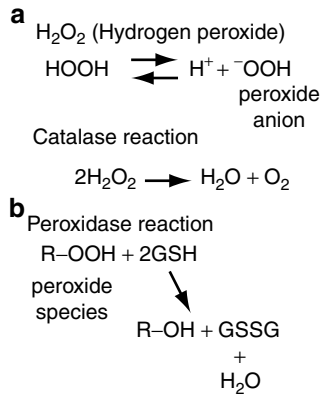


Fig. 16.8 Fluoride promotes retention of reactive oxygen species. The peroxide ion (^-OOH) is a reactive oxygen species derived from hydrogen peroxide or attached to an organic molecule (R^-). (a) Hydrogen peroxide is eliminated by catalase that converts it to oxygen gas and water. (b) Organic peroxide is eliminated by peroxidase converting it to the corresponding organic alcohol and water with assistance from glutathione (GSH), which loses its electrons and is oxidized (GSSG). Fluoride inhibits both enzymes, causing organic peroxides to accumulate, see text

Fluoride causes mottled enamel at 1 ppm, toxic effects on bone at >10 ppm and more general effects at >50 ppm. Fluoride poisoning is especially associated with a lack of energy due to its inhibiting enolase and therefore of glycolysis. If taken at high levels to control osteoporosis, it inhibits osteoblast activity more than osteoclast activity, resulting in an increased frequency of bone fractures. In the 1940s, a bauxite plant to extract aluminum in the Fort William area of rural west Scotland poisoned local cattle by emitting fluoride at 50–100 ppm. These high levels of fluoride inhibit gluconeogenesis by binding to Mg^{+2} ions that activate fructose biphosphate phosphatase and protein synthesis by inhibiting the Mg^{+2} ion association of ribosomal subunits during eukaryotic protein synthesis initiation. These high levels of fluoride also inhibit peroxide and other reactive oxygen species elimination by binding to iron, selenium and molybdenum ions at the respective catalytic centers of catalase, peroxidase, and superoxide dismutase. At levels of 1 ppm, fluoride shows none of these toxic effects and is safe and effective in the water supply and toothpastes to prevent caries.

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